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VERIFICATION

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The undersigned declares further that all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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[TITLE OF INVENTION] METHOD FOR SELECTIVELY SEPARATING LIVE CELLS EXPRESSING A SPECIFIC GENE

[WHAT IS CLAIMED IS]

[Claim 1]

A method for selectively separating live cells which have expressed a specific gene comprising:

a first step of introducing markers which label mRNA into cells in a live cell group containing live cells which have expressed a specific gene;

a second step of labeling said mRNA with said marker to obtain a live cell group containing live cells having the labeled mRNA; and

a third step of detecting said labeled mRNA to identify the live cells having said labeled mRNA and separating the identified live cells selectively from said live cell group obtained in said second step.

[Claim 2]

The method according to claim 1, wherein said marker in said first step is a probe which has a base sequence complementary to said mRNA and has been labeled with a fluorescence dye, said labeled mRNA in said second step is a hybrid of the probe and said mRNA, and the selective separation in said third step is performed by irradiating light to the live cell group containing live cells having the hybrid, identifying live cells which cause a change in fluorescence of said fluorescence dye based on formation of the hybrid, and separating the identified live cells from the live cell group.

[Claim 3]

The method according to claim 2, wherein said probe comprises a first

probe and a second probe, the first probe and the second probe have base sequences capable of hybridizing to said mRNA adjacently, the first probe is labeled with an energy donor fluorescence dye and the second probe is labeled with an energy acceptor fluorescence dye, and said change in fluorescence is fluorescent resonance energy transfer (FRET) from the energy donor fluorescence dye of the first probe to the energy acceptor fluorescence dye of the second probe.

[Claim 4]

The method according to claim 2 or 3, wherein the selective separation in said third step of said live cells which causes the change in a fluorescence is performed by a cell sorter (FACS).

[Claim 5]

The method according to any one of the claims 2 to 4, wherein said mRNA is mRNA encoding a cytokine.

[Claim 6]

The method according to any one of the claims 3 to 5, wherein said mRNA is an mRNA encoding interleukin-2 (IL-2), said first probe is a probe having a base sequence set forth in SEQ ID NO: 9 in the Sequence Listing, and said the second probe is a probe having a base sequence set forth in SEQ ID NO: 10 in the Sequence Listing.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[Technical Field to Which the Invention Belongs]

This invention relates to the method for selectively separating live cells which have expressed a specific gene.

[0002]

[Prior Art]

If translation products of said gene are cell surface molecules, methods for selectively separating cells which have expressed specific genes, which are kept alive, include, the method for binding fluorescent marker antibodies to the surface molecule to identify fluorescing cells by flow cytometers to separate the identified cells by cell sorters (Fluorescence Activated cell sorter, FACS). In addition, panning method is also known wherein only the objective cells are absorb over the bottom surface of a dish over which is covered with antibodies specifically binding with cell surface molecules cover.

[0003]

On the other hand, when it molecules are those which translation products predominantly localize in the cells (in the cytoplasm or in organs) without production of any cell surface molecules as translation products of a gene, the methods described above cannot be adopted. In this case, it is theoretically possible to fluoresce expressed cells by a micro-injection method by introducing fluorescent marker antibodies into molecules to the separate the objective gene expressed cells by cell sorters described above based on the difference of fluorescence intensities generated by irradiation of laser beam or the like.

[0004]

[Problems to be Solved by the Invention]

However, for cell labeling methods by micro-injection methods described above, the introduction of fluorescent marker antibodies to one or some of cells per experiment is a limit for the methodology. In addition, these methods can not label many cells, and it is not easy to introduce

antibodies with more than 120,000 of molecular weight into cells, because the polymers have higher viscosity. Therefore, there are problems to realize these methods.

[0005]

If translation products of genes are not cell surface molecules, and if translation products are liberated into the extracellular liquid and if they are molecules which do not stay in the cell or near the cell membrane, it is very difficult to selectively trap and separate cells, which have expressed a specific cell, from others with the approaches described above. This is because, during the process that polypeptide chains translated and generated based on the genetic information are folded and secreted extracelullarly, their structure changes gradually and from time to time, preventing antibodies from binding to the polypeptide chains efficiently.

[0006]

A typical example, which cause the problems described above, includes the case where cells secreting cytokines are selectively separated as translation products of genes.

[0007]

When an antigen penetrates an organism, the helper T cell (CD4+T cell) which the recognize the antigen as a foreign matter is activated, and then it is differentiated into TH1 and TH2, both of which have different immunological functions from each other: TH1 (T Helper 1) which activates macrophages to remove foreign matters by phagocytosis and has cellular immunological functions; and TH2 (T Helper 2) which activates B-Cells to produce antibody molecules to neutralize foreign matters and has liquid immunological functions (See Fig.44). TH1 and

TH2 as cytokines produce respectively interleukin-2 (IL-2) and interleukin-4 (IL-4). In the normal state, TH1 and TH2 control their functions each other and keep a balance. However, the broken relationship between TH1 and TH2 causes various infections or autoimmune disorders.

[0008]

To selectively separate and obtain TH1 or TH2 is medically more important, because functional cells are applied to the transplantation to an organism for immunological functions supplement. However, it is very difficult to selectively separate TH1 generating IL-2 and TH2 generating IL-4 by the conventional approaches, because there are no crucial cell surface antigens (markers) to identify TH1 and TH2, and because cytokines (IL-2 and IL-4) which TH1 and TH2 produce respectively do not stay in the cell or near the cell membrane, but they are liberated to the extracellular.

[0009]

In order to overcome the problems, the present invention is directed to provide the separation method which allows to selectively separate and obtain the objective cells, that is, the cells which have expressed specific genes, when there are not cell surface molecules usable as markers in the cell, when cells cannot distinguished from each other if present, or even when molecules to become markers are liberated to the extracellular liquid.

[0010]

[Means for Solving the Problems]

As a result that the present inventors devoted to the research, the conventional problems were found to be caused by the separation of gene expressed cell by using translation products of genes (polypeptide) as

targets (markers). According to the finding, the inventors have found it possible to selectively separate cells which have expressed specific genes and which are kept alive, when the targets (markers) are not translation products (polypeptide) but mRNA, which are transcriptional products of genes and mainly exist in cytoplasm on float, and used as targets (markers), if cell surface molecules usable as markers do not exist, if cells cannot be distinguished from each other if present, or even if molecules to become markers are liberated to the extracellular liquid. And then the present invention has been completed.

[0011]

That is, the present invention provides a method for selectively separating live cells which have expressed a specific gene comprising a first step of introducing a marker which labels mRNA into a cell in a live cell group containing a live cell which has expressed a specific mRNA, a second step of labeling said mRNA with said marker to obtain a live cell group containing live cells having a labeled mRNA, and a third step of detecting said labeled mRNA to identify the live cell having a labeled mRNA and separating the identified live cell selectively from said live cell group obtained in said second step.

[0012]

In the method for selectively separating live cells which have expressed a specific gene according to the present invention, it is preferable that the marker in said first step is a probe complementary to said mRNA and has been labeled with a fluorescence dye, said labeled mRNA in said second step is a hybrid of the probe and said mRNA, and the selective separation in said third step is done by irradiating a light to a

live cell group containing live cells having the hybrid, identifying live cells which cause a change in fluorescence of said fluorescence dye based on a formation of the hybrid, and separating the identified live cell from the live cell group.

[0013]

It is also preferable that said probe comprises a first probe and a second probe, the first probe and the second probe have base sequences capable of hybridizing to said mRNA adjacently, the first probe is labeled with an energy donor fluorescence dye and the second probe is labeled with an energy acceptor fluorescence dye, and said change in fluorescence is fluorescent resonance energy transfer (FRET) from the energy donor fluorescence dye of the first probe to the energy acceptor fluorescence dye of the second probe.

[0014]

In addition, in the method for selectively separating live cells which have expressed a specific genes according to the present invention, the selective separation in said third step of said live cells which cause the change in a fluorescence is preferably performed by a cell sorter (FACS).

[0015]

It is also preferable that said mRNA is mRNA encoding a cytokine; said mRNA is an mRNA encoding interleukin-2 (IL-2), said first probe is a probe having a base sequence set forth in SEQ ID NO: 9 in the Sequence Listing, and said the second probe is a probe having a base sequence set forth in SEQ ID NO: 10 in the Sequence Listing.

[0016]

[Specific Modes of the Invention]

A method for selectively separating live cells which have expressed a specific gene according to the present invention comprises a first step of introducing a marker which labels mRNA into cells in a live cell group containing live cells which have expressed a specific gene, a second step of labeling said mRNA with said marker to obtain a live cell group containing live cells having the labeled mRNA, and a third step of detecting said labeled mRNA to identify the live cells having the labeled mRNA and separating the identified live cells selectively from the said live cell group obtained in the said second step.

[0017]

Markers introduced into the cells in the present invention may be those which can label mRNA and not limited specifically. Although markers produces labeled mRNA when they bind to mRNA in the cells, if mRNA do not exist in the cells, or if markers are excessively introduced even when mRNA are present, markers which do not involve the bond with mRNA can remain in the cell. Then markers are preferably detectable only when they have been bound to mRNA, or those which can be detected to determine whether they have bound to mRNA or not.

[0018]

In the present invention, probes which have base sequences complementary to mRNA and are labeled by a fluorescent dye (hereinafter called fluorescence-labeled probe in some cases) as markers are preferably used. Although these probes form a hybrid with mRNA in the cell, as it is necessary to separate to detect probes which do not form a hybrid as described, it is preferable to use, as the present probes, those which cause fluorescent changes based on the formation of hybrid.

[0019]

For these probes, probes labeled with different fluorescent dyes from each other are preferably combined to use. In other words, it is preferred to use probes comprising the first and second probe, in which the first probe and the second probe have base sequence hybridizable with and adjacent to said the mRNA, the first probe labeled with an energy donor fluorescent dye, and the second probe labeled with an energy acceptor fluorescent dye.

[0020]

When the energy donor fluorescent dye which label the first probe and the energy acceptor fluorescent dye which label the second probe are brought close to each other with a proper distance (for example, less than 8nm), fluorescent resonance energy transfer (FRET) is possible. Therefore, marker positions of respective fluorescent dyes in probes are preferably placed with a distance which FRET occurs between energy donor fluorescent dyes and energy acceptor fluorescent dyes after the first probe, the second probe and the mRNA form a hybrid. The preferable distance between 2 types of fluorescent dye depends on the types of fluorescent dye and sites to hybridize in the mRNA. In general, however, the distance between the 2 fluorescent dyes is preferably within the length of 20 bases or less, more preferably within the length of 2-4 bases. When probes which can generate FRET are designed, for example, Lakowicz, J. R. "Principles of Fluorescence spectroscopy" (1983), Plenum press, New York can be referenced.

[0021]

Energy donor fluorescent dyes used in the present invention include 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-propionic acid and derivatives thereof (available as Bodipy 493/503 series from Molecular Probes Inc.); tetramethylrhodamine-5-(and-6)-isothiocyanate) and derivatives thereof available as TRITC series from Molecular Probes Inc.); and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid and derivatives thereof (available as Bodipy FL series from Molecular Probes Inc.)

[0022]

Energy acceptor fluorescent dyes used in the present invention include 1,1-bis(ε -carboxypenty1)-3,3,3',3'-tetramethyl indodicarbocyanine-5,5'-disulfonate potassium salt and derivatives thereof (available as Cy5 series from Amersham Pharmacia BioTech Inc.); 1,1'-bis(ε -carboxypentyl)-3,3,3',3'-tetramethyl indocarbocyanine-5,5'-disulfonate potassium salt) and derivatives thereof (available as Cy3 series from Amersham Pharmacia BioTech Inc.); X-rhodamine-5-(and-6)-isothiocyanate and derivatives thereof (available as XRITC series from Molecular Probes Inc.); 6-(((4,4-difluoro-5-(2-thieny1)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl) aminohexanoic acid and derivatives thereof (available from Bodipy 630/650 series from Molecular Probes Inc.); and 6-(((4,4-dilfluoro-5-(2-pyrolyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl) aminohexanoic acid and derivatives thereof (available as Bodipy 650/665 series from Molecular Probes Inc.)

[0023]

In the present invention, it is preferable to use Bodipy 493/503 series as an energy donor fluorescent dye, and Cy5 or XRITC series as

an energy acceptor fluorescent dye.

[0024]

In the present invention, the number of bases of oligonucleotide to form a probe is not strictly restricted. When the number of bases is extremely smaller, for example, when the number them is less than 10, however, it can be difficult to form a fully stable hybrid. When the number of bases in a probe is higher than 50 or so, not only the synthesis of a probe is difficult, but also the stability of the probe is degraded, and it can take a longer time for a hybrid formation.

[0025]

The number of bases in a probe is determined under the consideration of the conditions of hybridization, such as the concentration of target mRNA in a live cell to be used and the temperature in hybridization. Generally, the melting point of hybrid formed with a probe and mRNA is elevated with the increased number of bases in a probe. For example, if the number of bases in a probe is 15 or so, hybrids can be made at room temperature with adequately higher efficiency, but if at 37℃ the rate of the formation of hybrids are not always higher. In order to detect hybrids at 37℃, it is desirable to use probes with the length of 15 bases or more, preferably 20 bases or more.

[0026]

However, the tendency that the rate of hybrid formation decreases as the number of bases increases the same when the number of bases in probes is in the range of 15-20. For example, at room temperature, the time necessary to complete the hybridization of a probe of 20 bases and mRNA is several times longer than the time required for a probe of 15

bases is used. Taken these requirements together, it is desirable that the number of bases in a probe is 10-50, more preferably 15-20.

[0027]

While the number of bases in a probe becomes important when probes are designed as described, it is important what site in the mRNA probe hybridizes. That is, because generally, mRNA itself is a molecule with complicated three-dimensional structure, if a probe to be used has a base sequence complementary to a site of an mRNA, when the site interacts with other sites in the molecule, a hindrance to hybridization often occurs in the three-dimensional structure. In the present invention, therefore, it is preferable that a probe appropriately selects the site of mRNA to hybridize.

[0028]

The site to hybridize is determined, for example, by approaches described below. First, base sequences of the objective mRNA are obtained from databases. If databases are not available, the base sequences of the mRNA are determined by well-known methods. According to the information, secondary structures of mRNA are simulated. For this simulation, it is possible to use commercially available computer programs for predicting the structure of RNA, such as DNAsis (Hitachi Software Engineering Inc.). Using the obtained secondary structural drawings, proper numbers of base sequences mainly without hindrances in three-dimensional structures are selected, oligonucleotide having base sequences complementary to the selected base sequence is synthesized, the synthesized oligonucleotide is fluorescence-labeled, and the oligonucleotide is used as a probe.

[0029]

When a set of probes which can cause FRET is designed based on the three-dimensional structural drawings by computer programs, it is preferable that, mainly using parts with no hindrance to three-dimensional structures of mRNA, for example, several sites having 30-40 bases are selected, each site is subdivided into parts (each 15-20 bases), and oligonucleotide having base sequences respectively complementary to the subdivided parts is synthesized and fluorescence-labeled to use as the first probe and the second probe.

[0030]

In order to select an optimal set of probes from some sets of probes synthesized as above, the following method can be used: a mixed solution of the first probe and the second probe which have been synthesized by methods describe above is prepared to measure fluorescence spectra; then, the objective mRNA is added to the mixed solution to observe the fluorescence spectra. When the first probe, the second probe and mRNA is formed into a hybrid, FRET occurs between 2 types of fluorescent dyes; as a result, the fluorescence intensity of the energy donor dye decreases, while the fluorescence spectrum is obtained where the fluorescence intensity of the energy acceptor dye is increase; the operation described above is carried out to some types of sets of said first and second probes; the changes of fluorescence spectra are compared; a set of probes with greater change is selected. The objective mRNA used for these method can be synthesized by in vitro transcription reaction using recombinant plasmid DNA which includes cDNA corresponding to the mRNA.

[0031]

In order to evaluate the efficiency of hybridization to mRNA correctly, after each probe and each objective RNA are mixed and reacted to an aqueous solution, using high performance liquid chromatography (HPLC) or others, a hybrid and a free probe are separated to determine a ratio of a probe forming a hybrid to entire probes.

[0032]

The optimization of fluorescence-labeled probes has been described above in detailed. The fluorescence-labeled probes are preferable form of markers used in the present invention as described above. In the present invention, after markers have been prepared, these agents are introduced into the live cells which have expressed a specific gene. As there are no limits to the methods for introducing markers into the live cell, well-known methods are available including micro-injection, electroporation, and lipofection methods. In the present invention, an electroporation method is preferable, because the method can introduce marker agents into more than 10,000,000 of live cells in a short time at one time.

[0033]

After markers have been introduced into live cells, mRNA are labeled in the cells by the relevant markers. Fluorescence-labeled probes are used as marker, as the probes specifically hybridize with the corresponding mRNA. The conditions to hybridize are not limited specifically, but, for example, live cells which have been introduced fluorescence-labeled probes, must be retained at room temperature at least for a few minutes.

[0034]

After the cellular mRNA is labeled, live cells containing the labeled mRNA are identified by detecting the labeled mRNA, the identified cells are selectively separated. In the present invention, there are no limits for the procedure to detect the labeled mRNA. However, all of the live cell groups of which markers have been introduced do not always possess the objective mRNA. If the mRNA exists, when marker has been introduced excessively in amount, marker which does not bind to the mRNA will remain in the cell. Therefore, it is necessary to separate the labeled mRNA from unbound and free markers to detect the mRNA specifically.

[0035]

As a method for easily and highly sensitively detecting labeled mRNA in the presence of markers unbound to the mRNA, the first probes which are labeled with energy donor dyes and the second probes which are labeled with energy acceptor dyes are preferably used together: these probes are introduced into the live cells to keep the live cells under the conditions that the probes and the mRNA can hybridize, the excitation light of the energy donor fluorescent dyes of the first probes is irradiated to the live cell groups, whereby the fluorescence from the energy acceptor fluorescent dye of the second probe is observed based on FRET, and finally it is possible to detect the fluorescence-labeled mRNA specifically.

[0036]

Although the irradiation of excitation light excites the energy donor dyes of the first probes whether the probes hybridize with the objective mRNA or not, only when the both of the first and second probes hybridize adjacently with the same mRNA, FRET occurs, and at this time, fluorescence is generated from the energy acceptor fluorescent dyes. That

is, the FRET-fluorescence from the acceptor fluorescent dyes can be an index indicating that the first probe and the second probe are adjacent on the objective mRNA. Thus, it is found that the objective genes are expressed in the live cells based on the fluorescence-labeled mRNA.

[0037]

The live cells which have expressed specific genes detected in this way are selectively separated, but there is no limitations for this separating method. In the present invention, when fluorescence-labeled probes are used, it is preferable to use cell sorters (Fluorescence Activated cell sorter, FACS) to detect live cells, which express specific genes, and then to selectively separate them.

[0038]

In general, an apparatus consisting of a flow cytometer and a cell dispenser is called a cell sorter. Individual cell, which has been stained with fluorescence-labeled probes, is exposed to laser beam on the way of a flow path, whereby scattering light (forward scattering light or side scattering light) and signal information of fluorescence for every cells. The results are displayed, for example, as a frequency distribution diagram (dot plot). The cell sorter can dispense desired cells emitting specific fluorescence signal by gating. The method as mentioned above is called flow cytometry.

[0039]

In the present invention, when live cells which have expressed specific genes are selectively separated with fluorescence-labeled probes which can cause FRET, the following method can be applied, for example: obtaining relative fluorescence intensities of fluorescent dyes which are

determined from laser irradiation exciting energy donor fluorescent dyes (for example, Bodipy 493/503) in each live cells or fluorescent dye (for example, Cy5) based on FRET by a cell sorter, and signal information of relative fluorescence intensity of energy accept; dot plotting by setting the value of the intensities of the donor fluorescent dye and the acceptor fluorescent dye on the horizontal and the vertical axis, respectively; selecting a cell group of which signal level is higher; and gating the cell group (this gating condition is referred to as R2 herein). In addition, after forward scattering light based on live cells to be measured and signal information of side scattering light based on the complexities of the intrastructures of live cells are obtained, the regions which represent live cells to be measured are gated, for example, by dot plotting based on the values of forward scattering light and side scattering light on the horizontal and the vertical axis, respectively (this gating condition is referred to as R1 herein). After a cell sorter are set so that only live cells suitable for the both conditions of R1 and R2 can be dispensed, only the cells possessing specific mRNA can be selectively separated.

[0040]

By using the methods as described, various types of live cells as well as various type of mRNA are objectives of selective separation. TH1 and TH2 derived from helper T cells which have been recognized antigens as foreign matters and activated, do not have crucial cell surface antigens (markers) for identifying to each other, and cytokines produced by TH1 and TH2 (IL-2 and IL-4) do not stay in the cell or near the cell membrane and are liberated to the extracellular liquid, so that TH1 or TH2 is optimal to be applied to the selective separation in the present invention. That is,

the selective separation method according to the present invention is preferably used for live cell groups having mRNA encoding cytokines.

[0041]

Especially, it is preferable that live cell groups containing live cells having mRNA encoding interleukin-2 (IL-2) are intended to use with the first probes labeled by energy donor fluorescent dyes having base sequences of SEQ ID NO: 9 of the Sequence List and the second probes labeled by energy acceptor fluorescent dyes having base sequences of SEQ ID NO: 10 of the Sequence List, and FRET generated by these probes is utilized to selective separation.

[0042]

The base sequence of SEQ ID NO: 9 of the Sequence List is complementary to the base sequence between 342nd-356th bases in the base sequence of mRNA, while the base sequences of SEQ ID NO: 10 of the Sequence List is complementary to the base sequence between 357th-371st bases. When the first probes labeled with energy donor fluorescent dyes are made adjacent to the second probe labeled with energy acceptor fluorescent dyes on the positions of the same mRNA molecule as described above, the detection of the FRET-fluorescence can be high-sensitively carried out.

[0043]

[Examples]

Although this invention is concretely illustrated by way of examples, it is not limited to the following examples insofar as it does not depart from its essence.

[0044]

(1) Preparation of fluorescence-labeled probes complementary to IL-2 mRNA

After randomly selecting five parts of sequences, 30 consecutive bases each, from the entire base sequence of IL-2 mRNA, an oligo DNA probes were designed based on the sequences complementary to those of IL-2 mRNA and fluorescence-labeled with Bodipy493/503 (an energy donor fluorescent dye), Cy5 (an energy acceptor fluorescent dye), or XRITC (an energy acceptor fluorescent dye).

[0045]

The designed probes of nucleic acids were synthesized using a DNA/RNA synthesizer (Perkin Elmer: Model 394 or Perceptive: Model 18909), by β-cyanoethylamidite method. The entire base sequence of IL-2 mRNA and base sequences of oligo DNA probes are shown in Fig.1. The designed 10 types of base sequences of oligo DNA probes (SEQ ID No: 1-10) and the base number (hybridized positions) of IL-2 mRNA which the oligo DNA probes hybridize are shown in Table 1.

[0046]

(Table 1)

SEQ ID NO:	Base Sequence	Hybridized Position*
SEQ ID NO: 1	5'-GTAAAACTTAAATGT-3'	228-242
SEQ ID NO: 2	5'-GGCCTTCTTGGGCAT-3'	243-257
SEQ ID NO: 3	5'-TTTGGGATTCTTGTA-3'	198-212
SEQ ID NO: 4	5'-GAGCATCCTGGTGAG-3'	213-227
SEQ ID NO: 5	5'-GCAAGACTTAGTGCA-3'	77-91
SEQ ID NO: 6	5'-CTGTTTGTGACAAGT-3'	92-106
SEQ ID NO: 7	5'-GGTTTGAGTTCTTCT-3'	287-301
SEQ ID NO: 8	5'-AGCACTTCCTCCAGA-3'	302-316
SEQ ID NO: 9	5'-CCTGGGTCTTAAGTG-3'	342-356

SEQ ID NO: 10	5'-ATTGCTGATTAAGTC-3'	357-371

*:Base number of IL-2 mRNA to which each probe hybridizes.

[0047]

In item (4), probes, which 5' terminals of base sequences of SEQ ID NO: 1-10 as described were labeled with Bodipy493/503, are used, and in item (8), base sequences of SEQ ID NO: 1-10 as described were used as probes but they were not labeled. In item (9), probes, which 5' terminals of SEQ ID NO: 1, 3, 6, 7, and 9 as described, and probes, which positions between the 4th and 5th base sequences from 3' terminals of SEQ ID NO: 2, 4, 6, 8, and 10 as described were labeled with XRITC, were used. In item (11), probes, which 5' terminals of SEQ ID NO: 1, 3, 5, 7, and 9 were labeled with Bodipy493/503, and probes, which positions between the 4th and 5th base sequences from 3' terminals of SEQ ID NO: 2, 4, 6, 8, and 10 were labeled with Cy5, were used. Methods for labeling with Bodipy493/503, XRITC, or Cy5 were followed by the methods designated by (a)-(c), respectively.

[0048]

In the present invention, the oligo DNA probes, which are labeled with energy donor fluorescent dyes, are sometimes abbreviated as donor probes, while oligo DNA probes, which are labeled with energy acceptor fluorescent dyes are sometimes abbreviated as acceptor probes. As the probe, which the base sequence of SEQ ID NO: 1 was labeled with energy donor fluorescent dyes, is complementary to the base sequence between the 228th and 242nd bases of IL-2 mRNA, the probe is sometimes represented by 228-242(D) (D means a donor). The probe, which the base sequence of SEQ ID NO: 2 was labeled with energy

acceptor fluorescent dye, is complementary to the base sequence between the 243rd and 257th bases of IL-2 mRNA, therefore the probe is sometimes referred as 243-257(A) (A means an acceptor); however, if the probe is not labeled with a fluorescent dye, the probes are simply represented, for example, as 228-242. Thus, probes used in items (9) and (11) are represented by the names of probes shown in Table 2 hereunder.

[0049]

(Table 2)

SEQ ID NO:	Base sequence	Name of Probes
SEQ ID NO: 1	5'-GTAAAACTTAAATGT-3'	228-242(D)
SEQ ID NO: 2	5'-GGCCTTCTTGGGCAT-3'	243-257(A)
SEQ ID NO: 3	5'-TTTGGGATTCTTGTA-3'	198-212(D)
SEQ ID NO: 4	5'-GAGCATCCTGGTGAG-3'	213-227(A)
SEQ ID NO: 5	5'-GCAAGACTTAGTGCA-3'	77-91(D)
SEQ ID NO: 6	5'-CTGTTTGTGACAAGT-3'	92-106(A)
SEQ ID NO: 7	5'-GGTTTGAGTTCTTCT-3'	287-301(D)
SEQ ID NO: 8	5'-AGCACTTCCTCCAGA-3'	302-316(A)
SEQ ID NO: 9	5'-CCTGGGTCTTAAGTG-3'	342-356(D)
SEQ ID NO: 10	5'-ATTGCTGATTAAGTC-3'	357-371(A)

[0050]

(a)Preparation of Donor Probes (Bodipy 493/503 markers)

NHSS(N-Hydroxysulfosuccinimide sodium salt) of 2.5 mg in 30 μl of sterilized water and 5 mg of EDAC (1-ethyl-3- (3-dimethylamino propyl)carbodiimide) in 50 μ l of sterilized water were mixed and reacted with 1mg of Bodipy493/503 propionic acid in 50 μ l of DMF at room temperature for 30 minutes.

On the other hand, using 5' terminal aminating agent 6-

(trifluoroacetylamino)hexyl(2-cyanoethyl)-(N,N-di-isopropyl)-phosphoroamidited, a material which a hexylamino group was introduced into 5' terminal of oligo DNA with the base sequence described above (a lyophilized product) was dissolved in 200μl of 0.5 M of Na_2HCO_3/NaH_2CO_3 buffer solution (pH9.3).

[0051]

These were mixed and reacted overnight in the dark; the reacted solution was gel filtrated; unreacted dyes were removed; and a reaction product with absorption bands at 260nm and 493nm was dispensed with CAPCELL PACK18 (Shiseido Inc., Column size: 6mm in inner diameter × 250mm in total length) by reversed phase high performance liquid chromatography (Flow rate: 1ml/minute, temperature in column:40℃, composition in mobile phase: 5% solution A of CH3CN containing 5mM of TEAA and 40% solution B were prepared, and the solution B was loaded onto concentration gradient with the ratio of 30 – 80% for 20 minutes); and the dispensed product was lyophilized.

[0052]

(b) Preparation of Acceptor probes (Cy5 marker)

Cy5 pigments in one tube (Amersham Fluorolink Cat. No. PA25001) were dissolved in 100 μ l of sterilized water. On the other hand, using Uni-Link AminoModifier (Clontech Inc.), a composition which a hexylamino group was introduced into the site between the 4th and 5th bases from 3'terminal of oligo DNA having base sequences described above (a lyophilized product) was dissolved in 200 μl of Na2HCO3/NaH2CO3 buffer solution (0.5 M, pH9.3); these were mixed, protected from light, and reacted over night; the reacted solution was gel

filtrated; unreacted dyes were removed; a component with an absorption band at 260 nm was dispensed with CAPCELL PACK18(Shiseido Inc., Column size: 6m min inner diameter × 250 mm in total length) by reversed phase high performance liquid chromatography(Flow rate: 1 ml/minute, Temperature in column:40℃, Composition in mobile phase: 5% solution A of CH3CN containing 5 mM TEAA and 40% solution B were prepared, and the solution B was loaded onto concentration gradient with the ratio between 15 - 60% for 20 minutes concentration gradient); a component with an absorptive band at 260nm was dispensed; the absorption spectra were measured within the range of 220-700nm; and after the maximum absorptive band was observed between 650-700nm in Cy5, the dispensed component was lyophilized.

[0053]

(c) Preparation o f Acceptor Probes (XRITC marker)

100 µl of XRITC dye solution (Solvent: 100% DMS0, Perkin Elmer, ROX-NHS) was reacted with oligo DNA having base sequences described above which a hexylamino group was introduced as described in item (B). The reaction product was applied to reversed phase high performance liquid chromatography; a component with an absorptive band at 260nm was dispensed; the absorption spectra was measured with in the range between 220-650 nm; after the maximum absorption of XRITC was found with in the range between 550-600 n, the dispensed component was lyophilized.

[0054]

(2) In vitro Synthesis of human IL-2 RNA

In order to obtain human IL-2 RNA having base sequences equivalent to human IL-2 mRNA, plasmid DNA with a human IL-2 cDNA and IL-2 cDNA fragment cleaved out with restriction enzyme pst I from pTCGF-II (ATCC# 39673) were linked with a ligation kit (Takara Inc.) so that the cDNA would be located to the downstream of T3 promoter to the pst I digestion site of a plasmid vector pBluescript KS(+) for synthesizing. The obtained recombinant plasmid was introduced a competent cell of E. Coli JM109 strain (Takara Inc.); the transformants of the E. Coli obtained were cultured; 46.2 μ g of plasmid DNA was extracted and purified from 100 ml of the medium using the Plasmid Midi Kit(QIAGEN).

[0055]

The recombinant plasmid was linearized by restriction enzyme Sma I digestion; and after protein in the plasmid solution was decomposed with proteinase K and modified/removed with phenol/chloroform. Using the purified gene fragment (0.66 μ g) as a template, T3 RNA polymerase solution which the composition ratios of bases making up the RNA were A:C:G:U=3.5:1.8:1.4:3.2% were prepared with an in vitro transcription kit (Megascript T3 Kits, Ambion), the prepared material was polymerase - reacted at 37℃ for six hours, and human IL-2 RNA was synthesized which human IL-2 cDNA was used as a template. After the end of the reaction, the template DNA was decomposed with DNase I (Megascript T3 Kits, Ambion Inc.), protein in the transcriptional reaction solution was modified/removed with phenol/chloroform. To the obtained RNA solution was added isovolumic isopropanol, human IL-2 RNA was collected as a precipitate by centrifugation (14 krpm, for 7 minutes), substances of unreacted enzyme reactive substrate including each nucleotide. humanly-

2 RNA precipitates (139 μg) rinsed with 70% ethanol was dissolved with water free of RNase (Megascript T3 Kits, Ambion); 5 μ g/ μ l of human IL-2 RNA solution was prepared and used for hybridization experiments hereunder.

[0056]

(3) Changes of Fluorescent Spectra by hybridization of Fluorescent marker probes and human IL-2 RNA

In order to measure changes in fluorescence spectra based on fluorescent resonance energy transfer(FRET) associated with hybridization of donor probes and acceptor probes which are adjacent to each other, the combination of 300 nM (final concentration) of Bodipy 493/503 marker donor probes and XRITC marker acceptor probes, and human IL-2 RNA were mixed, used as 100 μ l of 1 × SSC solution (150mM sodium chloride, 17 mM citric acid, pH 7.0), and allowed to stand at room temperature for 15 minutes, and then fluorescence spectra was measured. As combinations of donor probes and acceptor probes, 228-242(D) and 243-257(A), 198-212(D) and 213-227(A), 77-91(D) and 92-106(A), 287-301(D) and 302-316(A), and 342-356(D) and 357-371(A). As a control, a fluorescence spectrum of 300 nM of single probe described above alone was also measured. The conditions for measurement were as follows:

Fluorospectrophotometer: F4500 (Hitachi)

Excitation wavelength; 480nm

Fluorescence-measured Wavelength: 500-750 nm

Temperature: room temperature

[0057]

As a result, when human IL-2 RNA were added to all of the

combination respectively, fluorescence intensities of donor dyes after energy donor fluorescent dyes were excited decreased by FRET, remarkable changes of fluorescence spectra (increase of fluorescence intensities) were found in the region of fluorescent wavelength energy acceptor dyes. Fig.2, 3, 4, 5, and 6 show fluorescence spectra when the combination of 228-242 (D) and 243-257(A), 198-212(D) and 213-227(A), 77-91(D) and 92-106(A), 287-301(D) and 302-316(A), and 342-356(D) and 357-371(A) were used. As seen from the comparison among Fig.2-6, fluorescence spectra were different from each other in the combinations of probes, the most remarkable change was found when the combination of 342-356(D) and 357-371(A) was used.

[0058]

(4) Measurement of Hybridization Efficiency of Probes and Human IL-2 RNA by HPLC

Each donor probes of 3 pmol which oligo DNA having base sequences of SEQ ID NO: 1-10 described above was labeled with Bodipy 493/503 markers was mixed with the same mol of human IL-2 RNA synthesized in item (3); each mixture was made into 10μl of 1XSSC solution (150 mM sodium chloride,17 mM sodium citrate, pH 7.0); each was allowed to stand at room temperature for fifteen minutes. Hybrids consisting of human IL-2 RNA and probes described above were separated respectively by high performance liquid chromatography(HPLC) from free probes using differences in retention time under the following conditions: (retention time under the same condition is about 4-5 minutes for probes and about 7.5 minutes for hybrids).

Column: TSKgel DEAE-NPR(Toso Inc., 4.6 mm in inner

Diameter × 35 mm in total length)

Column Flow Rate: 1 ml/ minute

Column Temperature: 25℃

Mobile phase: Solution A: 20 mM Tris-HCl(pH9.0)

Mobile phase: Solution B:0.5 M NaCl, 20 mM TrisHCl (pH9.0)

[0059]

Concentration gradient was loaded onto the ratio of the solution B to the solution A between 25-100% (corresponding to 0.125-0.5 M NaCl) for 10 minutes; the detection of peaks for nucleic acid by ultraviolet absorption (260nm) to eluted components and peaks for marker dyes Bodipy493/503 at excitation wavelength of 475nm/emission wavelength of 515 nm was carried out simultaneously, the ratio of fluorescent peak area of the hybrid to the entire area was determined and used as an index for hybridization efficiency.

[0060]

Fig.7 shows a HPLC chromatogram when 342-356 (D) was used. Fig.8 shows a HPLC chromatogram when 357-371(D) was used. Ratios based on peak areas of hybrids for probe were shown in Table 3, respectively and together.

[0061]

(Table 3)

Name of Probe	Base Sequence	Ratio of Hybrid(%)
228-242(D)	5'-GTAAAACTTAAATGT-3'	0.1

243-257(D)	5'-GGCCTTCTTGGGCAT-3'	17.5
198-212(D)	5'-TTTGGGATTCTTGTA-3'	15.7
213-227(D)	5'-GAGCATCCTGGTGAG-3'	25.2
77-91(D)	5'-GCAAGACTTAGTGCA-3'	0.5
92-106(D)	5'-CTGTTTGTGACAAGT-3'	18.3
287-301(D)	5'-GGTTTGAGTTCTTCT-3'	13.3
302-316(D)	5'-AGCACTTCCTCCAGA-3'	6.2
342-356(D)	5'-CCTGGGTCTTAAGTG-3'	22.8
357-371(D)	5'-ATTGCTGATTAAGTC-3'	27.3

[0062]

From the results shown in Table 3, it was found that the individual probes of 213-227(D), 342-356(D), and 357-371(D) hybridized to the target RNA more effectively. In item (3), 342-356(D) and 357-371(A) showed the most remarkable changes in fluorescence spectra as donor probes or acceptor probes which affect the efficiency of hybridization, therefore the results of the experiments in items (3) and (4) were considered to be consistent.

[0063]

(5) Induction of IL-2 gene expression in Human T-Cell leukemia strain cells Jurkat E6-1

To Jurkat E6-1 with a cell density of 1×10^6/ml was added 0.5 mg/ml (final concentration) of anti-CD3 antibodies (available from Immunotech Inc.), the same amount of anti-CD28 antibodies (available from Immunotech Inc.), and 10 nM PMA(available from Sigma Inc.) and cultured for three days (24 hours) at 37℃ under the presence of 5% carbon dioxide.

[0064]

(6) Measurement of the amount of IL-2 Protein Molecules

If more IL-2 molecules are produced and released into culture supernatant depending on the induction treatment of IL-2 gene expression in item (5), IL-2 mRNA in the cells is regarded to be synthesized actively. Thus, in order to confirm IL-2 gene expression, culture supernatant of Jurkat E6-1 cells (in some cases, hereinafter called IL-2 expression-induced cell) which were treated in the way of item (5) was collected; the amount of IL-2 (pg/ml/107 cells) in the supernatant were determined by ELISA sandwich method using a human interleukin-2 measurement kit; and the cells were compared with untreated cells (in some cases, hereinafter called IL-2 expression-uninduced cells).

[0065]

Wells with 96 well plates(antibodies plate) on which anti-human IL-2 monoclonal antibodies were solidified were washed with washing solution twice, and 150 μl of buffer solution was added to each well to be used. To each well was added 50 μl of standard IL-2 (0-1,600 pg/ml, an accessory of said measurement kit) and the culture supernatant described above, and the they were incubated at 37℃ overnight. The reacted solution in each well was removed, and the reminders were washed with washing solution three times. The first antibodies (anti-human IL-2 rabbit serum) solution was added by 100 µl/well, and incubated at room temperature for 2 hours. The solution of antibodies in each well was removed, and washed with washing solution three times.

[0066]

The second antibodies (peroxidase labeled anti-rabbit IgG antibodies) solution was added by 100 µl/well, and incubated at room

temperature for 2 hours. The solution of antibodies in each well was removed; the reminder was washed three times; and each well was fully dried. 0.015% of hydrogen peroxide aqueous solution of peroxidase substrate (o-phenylenediamine) was added by 100 μl/well, and reacted at room temperature for 10-20 minutes. A reaction stop solution (1 N H2SO4) was added to each 100 μl each well to stop reaction. Absorbance at 492 nm in each well was measured using a microplate reader. IL-2 in the culture supernatant was determined based on the calibration curve created from the values of absorbance of Standard IL-2.

[0067]

Consequently, for IL-2 expression-induced cells, 6157±168 (pg/ml/10^7 cells) of IL-2 molecules were detected in the media, while IL-2 molecules in the supernatant were under the limit of detection for IL-2 expression-uninduced cells (<0.1pg/ml/10^7 cells).

[0068]

(7) Measurement of Amounts of IL-2 Gene Expression

For experimental materials, this experiment needed IL-2 RNA as standard, entire RNA extracted from Jurkat B6-1 cells as a measurement sample, and IL-2 RNA probe (labeled with digoxigenin) as a probe for IL-2 RNA or mRNA detection. They were obtained using the methods in the following items (a)-(c).

[0069]

(a) Standard IL-2 RNA

For standardIL-2 RNAs,human IL-2 RNAs(1 μg/μl) synthesized in item (2) were diluted with 1Xdilution buffer (sterilized distilled water free of RNase :20 ヲ SSC: formamide = 5:3:1) to levels (10^4, 10^5, 10^6, 10^7) in turn,

the diluted RNA solutions were heated at 68□℃ for ten minutes, then quenched, and used for blotting.

[0070]

(b) Entire RNA of Jurkat E6-1 cells.

Entire RNA of Jurkat E6-1cell was extracted using RNeasy kit(QIAGEN Inc.). Under the condition shown in item (5), cells treated for inducing IL-2 expression for 0, 24, 48, 72, and 96 hours (0.8-1.2$\times 10^7$cells) were recvered as precipitate by centrifugation at 1,500rpm for 5 minutes; the cells were suspend with 1,000 μl of homogenization buffer including 10 μl of β-mercaptethanol, aspirated and blown out with syringes with 18-gauges, and denatured sufficiently. To the same homogenate was added 1,000 μ l of 70% ethanol, then applied to a column for RNA absorption, centrifuged at 4,000×g for 5 minutes, and then added a buffer for washing and centrifuged to wash the column.

[0071]

To the column sterilized distilled RNase-free water was added, and the absorbed RNA was eluted. To the eluted RNA solution 0.1 times volume of 4M sodium acetate and the equal volume of isopropanol were added; then RNA was centrifuged at 15,000×g for 15 minutes and recovered as precipitates. After the RNA was dissolved in the sterilized distilled water without RNase, it was diluted with isovolumic 2×dilution buffer (sterilized distilled RNase-free water:20×SSC:formamide=1:6:2), heated at 68℃ and quenched for 10 minutes.

[0072]

(c) Digoxigenin (DIG) Labeled IL-2 RNA-probe

DIG marker IL2 RNA-probe was synthesized using DIG RNA

Labeling kit (Boehringer Mannheim Inc.). After removing protein denatured by phenol/chloroform, 10 μg of human IL-2 cDNA recombinant plasmid (pTCGF#2) DNA completely linearized by EcoRI digestion was purified by ethanol precipitation, and used as a template for RNA probe synthesis. The template DNA (5 μg) and 1.8 mM ATP, 0.9mM CTP, 0.7mM GTP, 1.1mM UTP, and 0.58 mM UTP(DIG marker) were mixed in the presence of T7 RNA polymerase; the mixture was incubated at 37℃ for 2 hours; DNase I solution was added and reacted for ten minutes; and the template DNA was decomposed. To the reaction solution were added 0.1 times volume of 5 M sodium acetate and one time volume of isopropanol, the synthesized RNA was recovered by centrifugation at 15,000×g for 15 minutes. The same RNA was dissolved in sterilized distilled RNase-free water.

[0073]

(d) Measurement of amounts of IL-2 gene expression

Using the materials obtained from items (a)-(c), the amount of IL-2 mRNA was measured. The cellular RNA sample solution and standard IL-2 RNA solution were dotted to a nylon membrane, washed with 5×SSC twice, the RNA on the nylon membrane was fixed using UV-Crosslinker (Biorad Inc.). The same nylon membrane, prehybridization buffer (5× SSC, 5% SDS, 50 mM sodium phosphate(pH7.0), 50% formamide, 2% Blocking Reagent (Boehringer Mannheim Inc.), and 1% N-lauryl sarcosinate were enclosed into a HybriBag (Inouchi Inc., Hot water resistant bag: L) and prehybridized at 68℃ for one hour.

[0074]

DIG labeled IL-2 RNA probe was diluted by prehybridization buffer

up to 100 ng/ml, boiled for ten minutes and then quenched, and used as a hybridization solution. Prehybridization liquid in the hybripack was replaced with hybridization liquid, and hybridized at 68℃, overnight. Nylon membrane was washed with 2×Washing solution (2×SSC, 0.1% SDS) twice for 5 minutes in each, and with 0.2×Washing solution (0.2×SSC, 0.1% SDS) at 68℃ twice for 15 minutes in each. After washed with Buffer I (100 mM of maleic acid, 150 M NaCl(pH7.5)) for one minutes, the nylon membrane was blocked with Buffer I (which Blocking Reagent(Boehringer Mannheim Inc.) was made into 1% solution with buffer I).

[0075]

Chemiluminescence of DIG-labeled RNA hybrids was detected with a DIG Luminescent Detection kit (Boehringer Mannheim Inc.). With Buffer II solution containing alkaline phosphatase (150 mU/ml) labeled anti-DIG antibodies, the nylon membrane was treated at room temperature for one hour, then washed with Buffer I twice for 15 minutes in each, and packed into a bag (LIFETECHNOLOGIES Inc., Photogene development folder) with 250 μ M of substrate solution which CSPD (disodium- (4-methoxyspiro [1,2-dioxetane-3,2'-(5'-chioro) tricyclo [3.3.1.13,7] decan] -4-yl) phenylphosphate) was diluted with Buffer III (0.1 M Tris,pH 9.7,0.1 M NaCl,0.05 M $MgCl_2$). After the photon number generated based on the labeled RNA was counted by Arugas 50 (Hamamatsu Photonics), a calibration curve was created according to the photon number of standard IL-2 RNA; and based on the number, IL-2 mRNA of total cellular RNA was determined. From the amount of IL-2 mRNA (mol) obtained and the number of cells used for extraction of the total cellular RNA, the number of

IL-2 mRNA molecules per single cell was determined (Fig.9).

[0076]

As shown in Fig.9, the number of IL-2 mRNA molecules in the single cell which the cells were treated for inducing expression for 0, 24, 48, 72, and 96 hours respectively were <0.29, $(0.76\pm0.17)\times10^4$, $(1.11\pm0.40)\times10^4$, $(1.22\pm0.67)\times10^4$, and $(1.20\pm0.28)\times10^4$, respectively; the cells which treated for 72 hours (3 days) were found to possess the most IL-2 mRNA molecules. In addition, as the contents of IL-2 (pg/ml/107 cells) in the media treated for the periods as described were <0.32, 1032 ±25, 2433±533, 2688±194, 2531±283 respectively, it was suggested that more IL-2 molecules were secreted associated with more activity of expression of IL-2 gene.

[0077]

(8) Intracellular Hybridization of each probe with IL-2 mRNA of human T-cell leukemia cells Jurkat E6-1 inducibly expressing IL-2 gene

IL-2 expression-induced cells which was prepared by the treatment with anti-CD 3 antibodies, anti-CD 28 antibodies, and PMA for 3 days as described in item (5), and IL-2 expression-uninduced cells, the hybridization between cellular IL-2 mRNA and each IL-2 probe was examined by IST(In Situ Transcription) as follows.

[0078]

The IL-2 expression-induced and -uninduced Jurkat E6-1 cells (5×10^5 cells/ml PBS(-)) were washed three times, and suspended with 1 ml of PBS(-), and mounted on 12 mm cover glass (poly-L-lysine was coated on the bottom); then a single layer of cells were prepared. Cell membranes were provided for material permeability by exposition to 0.5% Triton-X100

solution for 90 seconds at room temperature. The cells were quickly washed with PBS(-),10 μ M (final concentration) of probes having base sequences of SEQ ID NO: 1-10 in Table 1 (unlabeled with dye), oligo dT (deoxythymidine oligonucleotide, unlabeled with dye) or oligo dA (deoxyadnine oligonucleotide, unlabeled with dye) were added, and incubated for one hour at room temperature. The cells were washed with PBS(-) quickly, and fixed by 4% of paraformaldehyde solution at room temperature for 15 minutes.

[0079]

The cells were washed with 1×SSC three times; 1 mM deoxyribonucleotide solution(Boehringer Mannheim Inc.) containing 0.35 M of DIG(digoxigenin) marker dUTP and 1u/μ l reverse transcriptase (Toyobo, Inc.) were added to the cells; and they were incubated for 2 hours at 30℃. The cells were washed with 1×SSC three times, and treated with Blocking buffer (which Blocking Reagent(Boehringer MannheimInc.) was dissolved in maleic acid buffer solution so that its ratio would be equal to 1(w/v)%). The cells were washed with maleic acid buffer solution three times; FITC (Fluorescein-isothiocyanate) labeled anti-DIG antibodies (which was diluted with Blocking buffer up to 1 μg/ml) was added; and incubation was carried out for 30 minutes at room temperature. The cover glass was washed with PBS(-) three times, observed by fluorescence microscope, and the total fluorescence intensity per visual field (relative value) was measured.

[0080]

Fig.10 and 11 show the fluorescence micrographs obtained then. Fig.10 shows a fluorescence micrograph where composites formed

between all the cellular mRNA and oligo dT in the fixed cells of IL-2 expression-induced cells in the fixed state and IL-2 expression-uninduced cells were fluorescence-detected; this figure also shows the control experiments in the case where oligo dA was added instead of oligo dT, or where both DIG-labeled dUTP and reverse transcriptase were not added. Fig.11 shows a fluorescence micrograph, where a hybrid formed between IL-2 mRNA in the dells of IL-2 expression-induced cells in the fixed state and IL-2 expression-uninduced cells and a probe (fluorescence-unlabeled) as described. In Fig.11, two fluorescent images for each probe are shown in IL-2 expression-induced and -uninduced cells. Fig.12 shows the standardized value of each probe based on the fluorescence intensity (100%) when oligo dT was added after, the fluorescent intensity per one cell was calculated from the fluorescence intensities (mean value $\pm$ SE) and the number of cells per the visual field.

[0081]

As a result, 342-356 and 357-371 showed to hybridize with intracellular IL-2 mRNA the most efficiently and individually. As these are complimentary to the 30 consecutive base sequences in IL-2 mRNA molecule, it was suggested that if each was labeled with energy donor fluorescent dye or energy acceptor fluorescent dye, fluorescence based on FRET (fluorescent resonance energy transfer) associated with intracellular hybridization could be able to detect specifically.

[0082]

(9) Intracellular hybridization (ISH) of Donor Probes and Acceptor Probes with IL-2 mRNA in IL-2 Expression-induced Cells

In the result of the experiment by IST in item (8), we took notice

that probes were introduced into the cells in the visual field of a fluorescence microscope almost uniformly. This method for introducing probes was also applied to fluorescent labeling probe of donors and acceptors; and hybridization of these probes and intracellular IL-2 mRNA was measured bas fluorescence based on FRET; and each set of probes were compared and reviewed. As this method was developed with the tips from IST, it is called ISH (In Situ Hybridization).

[0083]

The solution of 2 μM (final concentration) of donor probes labeled with Bodipy 493/503 and acceptor probes labeled with XRITC was added to (8) IL-2 expression-induced cell and IL-2 expression-uninduced cell provided for material permeability to the surfaces of cell membranes as described for one hour at room temperature, and they were incubated. As sets of donor probes and acceptor probes, 228-242(D) and 243-257(A), 198-212(D) and 213-227(A), 77-91(D) and 92-106(A), 287-301(D) and 302-316(A), and 342-356(D) and 357-371(A) were used. Fluorescence-labeled probes were washed and removed with PBS(-), and the cells were fixed with , 4% of paraformaldehyde solution for 15 minutes, at room temperature. The cover glass was washed with PBS(-) three times, and fluorescence microscopy was executed. Fluorescence of A generated from the cells when the excitation light of A (energy acceptor dyes) was irradiated to the cells (in some cases, hereinafter called A/A image), fluorescence of D generated the cells from when excitation light of D (energy donor dyes) was irradiated to the cells (hereunder, sometimes called D/D image), and fluorescence of A emitted from the cells by fluorescent resonance energy transfer(FRET) when excitation light of D

was irradiated to the cells (in some cases, hereinafter called D/A image) were obtained. As a result, the maximum level of fluorescence based on FRET was obtained when 342-356(D) was used as a donor probe and when 357-371(A) was used as an acceptor probe. This fluorescence micrograph was shown in Fig.13. In Fig.13, two A/A images, two D/D images, and two D/A images of each set of probes are shown for IL-2 expression-induced and -uninduced cells. In this figure, results when probes were not introduced for control experiment are also shown.

[0084]

Next, D/A(fluorescence of A based on FRET by excited D) and A/A (fluorescence of A based on FRET by excited A) per one visual fielded of fluorescent image were measured; fluorescence intensity values (mean value$\pm$SE) per one cell were calculated from these measured values and the number of cells per one visual field. In order to determine the efficiency of intracellular hybridization, {(D/A per cell)/ (A/A per cell)} representing the ratio of the hybridized acceptor probes to the total probes per a single cell was worked out. In Fig.14, using this (D/A)/(A/A)(%) as an index, the results that each set of probes was compared are shown. When the results in items (8) and (9) are put in one shape, it is suggested that 342-356(D) and 357-371(A) hybridize to target mRNA individually and successively in the cells.

[0085]

<u>(10) Intracellular hybridization when donor probes and acceptor probes were introduced live IL-2 expression-induced cells</u>

Based on the results of (3)-(9), 342-356(D) and 357-371(A) were selected as probes for detecting IL-2 mRNA in the live cells. These

probes labeled with fluorescent dyes were introduced into live cells which expressed IL-2 genes; and the specific detection of hybridization was tried using the change of fluorescence by FRET as an index.

[0086]

Similar to the way in item (5), IL-2 expression-induced cells which were prepared by the treatment with anti-CD3 antibodies, anti-CD28 antibodies, and PMA for three days, and IL-2 expression-uninduced cells were recovered, washed with ice cooled PBS(-) twice, and suspended with PBS(-) adjusting the cell density to 1×10^7 cells/ml. The cell suspension of 0.9 ml was put into a cuvette for electroporation; and donor probes 342-356(D) labeled with Bodipy 493/503 of 5.4 nmol(final concentration: 6.0 μ M) and 5.3 nmol of (final concentration: 5.86 μ M) of XRITC-labeled acceptor probes 357-371(A)were added; and the cells were pulsed at 250V, 975 μF. After the cell suspension liquid was passed through 70 μ m of cell strainer (Falcon) and centrifuged mildly, the cells were resuspended with PBS(-). Further the passed liquid was re-passed through 40 μ m of strainer (Falcon Inc.); the re-passed liquid was centrifuged followed by resuspension to remove debris including dead cells as much as possible; and the reminder was observed with a fluorescence microscope.

[0087]

The results were shown in Fig.15. Fig.15 shows A/A, D/D, and D/A images, and the corresponding phase contrast micrographs of IL-2 expression-induced and uninduced cells. In Fig.15, two A/A images, two D/D images, two D/A images of each set of probes, and two phase contrast micrographs were shown individually. 1-3 cells out of 20-22 cells

were D/A-positive cells in the visual field for IL-2 expression-induced cells, suggesting that the donor probe and the acceptor probe adjacently hybridize with IL-2 mRNA. On the other hand, no D/A-positive cells were observed in the visual field for IL-2 expression-uninduced cells.

[0088]

(11) Selective Separation of cells which have expressed IL-2 genes by flow cytometry

Utilizing the differenciated intensities of FRET -fluorescence between IL-2 expressing and unexpressing cells based on the specific hybrid formation among the donor probe, the acceptor probe, and IL-2 mRNA, it was attempted that IL-2 expressing cells were selectively separated from unexpressing ones as follows.

[0089]

The cell suspension liquids of IL-2 expression-induced cells prepared in the same way as shown in item (9) and IL-2 expression-uninduced cells were mixed with the ratios of 100:0, 0:100, 50:50, and 20:80; and 0.9 ml of the cell suspension was tra into a cuvette; a donor probe, 342-356(D) labeled with Bodipy493/503 of 16.2 nmol (final concentration:18.0 □μM) and 14.7 nmol (final concentration: 16.4 μM) of a Cy5 labeled acceptor probe 357-371 (A) were added; they were pulsed under the same conditions as those in item (9). Live cells were collected to inject into an experimental device for flow cytometry (FACSCalibur, BECTON DICKINSON Inc.).

[0090]

In a part of flow path, relative fluorescence intensity (FL3-Height) generated from acceptors (Cy5 markers) based on said hybridization

when excitation light of energy donor fluorescent dye (Bodipy493/503) was irradiated to the cell was detected as dot-plots with relative fluorescence intensity (FL1-Height) by Bodipy493/503. Among these plots, a group of dots with the highest value of FL3-Height representing a group of fluorescing cells based on the hybridization, was designated as R2. On the other hand, the group of dots, representing the cell size (FSC-Height; forward scattering light) as well as the complexity in the intrastructure (SSC-Height; side scattering light) as typical human lymphoid cells was designated as R1 according to the reference value (FACSCalibur Training Manual, BECTON DICKINSON).

[0091]

The obtained dot-plots were shown in Fig.16-23. Fig.16 shows a dot-plot based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 100 to 0. Fig.17 shows a dot-plot with the same mixing ratio based on FL1-Height and FL3-Height. Fig.18 shows a dot-plot based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 0 to 100. Fig.19 shows a dot-plot based on the FL1-Height and FL3-Height with the same ratio. Fig.20 shows a dot-plot based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 50 to 50. Fig.21 shows dot plots based on FL1-Height and FL3-Height with the same ratio. Fig.22 shows a dot-plot based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 20 to 80. Fig.23 shows a dot-plot based on FL1-Height and FL3-Height with the same ratio.

[0092]

A cell group suitable for both R1 and R2 was separately dispensed by a cell sorting function (a cell sorter). The dispensed cell group was injected into FACSCalibur to detect as similar dot-plots in order to confirm that the group was still fluorescence-labeled as desired. The obtained dot-plots were shown in Fig.24-29. Fig.24 shows a dot-plot based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 100 to 0. Fig.25 shows a dot-plot based on FL1-Height and FL3-Height regarding the same mixing ratio. Fig.26 shows a dot-plot based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 50 to 50. Fig.27 shows dot plots based on FL1-Height and FL3-Height regarding the same mixing ratio. Fig.28 shows a dot-plot when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 20 to 80. Fig.29 shows a dot-plot based on FL1-Height and FL3-Height regarding the same ratio.

[0093]

The comparison of Fig.17, Fig.21, and Fig.23 revealed that the relative number of dots representing the cell group suitable for R2 to the entire number of dots decreased in relation to the decrease in the ratio of IL-2 expression-induced cells in the mixture from 100, 50 to 20 %, while the value of FL3-Height was totally background level regarding IL-2 expression-uninduced cells even when the same donor and acceptor probes were introduced to the cells (Fig.19). As most of the sorted-out cells by the selective separation were suitable for both R1 and R2, IL-2 expressing cells were found to be dispensed as the live cells emitting considerable FRET-fluorescence (see Fig.24-29).

[0094]

(12) The Comparison of Cell Groups before and after Flow Cytometry by Fluorescence Microscope

Parts of cells before and after flow cytometry were transferred to a glass-bottomed dish, and ratios of fluorescing cells of D/A, A/A, and D/D of the cells in the visual fields were examined.

[0095]

The results were shown in Fig.30-36. Fig.30, 32, 34, and 36 show fluorescent images of acceptor dyes based on FRET representing hybrid formation among IL-2 mRNA, donor probes and acceptor probes (D/A image) in the cells, fluorescent images of donor dyes by the donor-excitation representing donor probes in the cells (D/D image), fluorescent images of acceptor dyes by the acceptor-excitation representing the presence of acceptor probes in the cells (A/A image), and the corresponding phase contrast micrographs before flow cytometry. Fig.31, 33 and 35 show images of the cells after flow cytometry (selectively dispensed cells by the cell sorting function) as described above. In Fig.30-36, arrows to indicate parts of cells are shown in order to align the positions of cells between the fluorescent images and the phase contrast micrographs.

[0096]

Cell groups in Fig.30 and 31 were IL-2 expression-induced cells and IL-2 expression-uninduced cells (both of which were alive) mixed with the ratio of 100 to 0; cell groups in Fig.32 and 33 were those with the ratio of 50 to 50. Cell groups in Fig.34 and 35 were IL-2 expression-induced cells and IL-2 expression-uninduced cells (both of which were alive) mixed with the ratio of 20 to 80; cell groups in Fig.36 were those with the ratio of

0 to 100.

In Fig.30, the description of 20 cells in the phase contrast micrograph represents that there were 20 cells in the entire visual field. The descriptions of 5 cells, 4 cells and 7 cells in A/A, D/A and D/D image represent the number of the cells emitting A/A, D/A, and D/D-fluorescence were 5, 4, and 7, respectively.

[0097]

In Fig.32, the descriptions of 20 cells, 3 cells, 1 cell and 10 cells represent that the number of the cells overall visual field was 20 and the numbers of fluorescing cells of A/A, D/A, and D/D were 3, 1, and 10 respectively. In Fig.34, the descriptions of 36 cells, 7 cells, 1 cell and 20 cells represent that the number of the cells overall visual field was 36 and the numbers of fluorescing cells of A/A, D/A, and D/D were 7, 1, and 20, respectively. In Fig.36 the images of IL-2 expression uninduced cells, the descriptions of 21 cells, 2 cells, 0 cell and 3 cells represent that the number of the cells overall visual field was 21 and the numbers of fluorescing cells of A/A, D/A, and D/D were 2, 0, and 3 respectively.

[0098]

Thus, as the ratios of IL-2 expression-induced cells were decreased from 100, 50, to 20 %, it can be found that the relative number of D/A-fluorescing cells possessing fluorescence-labeled IL-2 mRNA to the cells in the entire visual field decreased.

[0099]

Fig.31 shows fluorescence images of the cells selectively separated from the mixture of IL-2 expression-induced and -uninduced cells (both of which were alive) with the ratio of 100 to 0. The description

of 7 cells in the phase contrast micrograph represents that 7 cells exist in the entire visual fields. The descriptions of 7 cells, 7 cells, and 7 cells in the A/A, D/A and D/D image represent the numbers of fluorescing cells of A/A, D/A and D/D were all 7.

[0100]

In Fig.32, all the descriptions of 6 cells represent that the number of the cells overall visual field was 6 and the numbers of fluorescing cells of A/A, D/A, and D/D were all 6. Similarly in Fig.35, the all descriptions of 5 cells, the number of the cells overall visual field was 5; and the numbers of fluorescing cells of A/A, D/A, and D/D were all 5.

[0101]

The comparison between Fig.30 and 31, Fig.32 and 33, Fig.34 and 35, it was found that only the cells in which IL-2 mRNA was fluorescence-labeled could be selectively separated by the cell sorting part of flow cytometry (a cell sorter).

[0102]

(13) Comparison between the Cells before and after Flow Cytometry by In Situ Hybridization

Parts of cells before and after flow cytometry were transferred to a glass-bottomed dish, and fixed with 4% of paraformaldehyde /PBS (pH7.4) at room temperature for 30 minutes. The number of cells expressing had IL-2 mRNA in the entire visual field (Fluorescent in situ Hybridization) was determined. First, in this FISH, in order to prevent the higher background caused by the remains of RNA probes in the cell after the wash-out procedure, IL-2 RNA probes, which were synthesized according to the method in item (7) (c), purified and

fragmented finely as shown in the following method, and the products of the fragmentation were used for hybridization experiments.

[0103]

RNA probes of 10 μg were dissolved in 100 μl solution for alkaline denaturation (42 mM NaHCO3, 63 mM Na2CO3, 5 mm DTT), incubated at 60℃ for 10-15 minutes; and 10 μl of 3M sodium acetate and 350 μl of ethanol were added to precipitate RNA probes. After cooled at -20 ℃ for 30 minutes, they were centrifuged at 16 krpm for 20 minutes. The obtained precipitates were washed with 70% ethanol and dried to dissolve in 50 μl of sterilized distilled RNase-free water, and then used as alkaline denatured of IL-2 RNA probe solution.

[0104]

Fixed cells to the bottom surface of a dish were washed with PBS(-) three times and treated with 0.1% Triton X-100/PBS solution at room temperature for 5 minutes; providing cell membranes material permeability; and the cells were washed with PBS(-) three times and treated with 0.2 N HCl at room temperature for 10 minutes. After the monolayer of cells was washed with PBS(-), they were incubated with 1□μg/ml of proteinase K/ PBS solution for 5 minutes at 37℃. After the monolayer was washed with PBS(-), they were fixed again with 4% of paraformaldehyde / PBS (pH7.4) for 30 minutes. The fixed cells were washed with 2 mg/ml of glycine/PBS twice (15 minutes/time), and treated with 50% of deionized formamide/2×SSC solution (solution A, described hereunder) for 30 minutes; the hybridization solution was (50% deionized formamide, 5×denhardt, 2×SSC, alkarine denatured IL-2 RNA-probe (1 μg/ml)) denatured at 90℃ for ten minutes and then cooled down; and the

monolayer was treated with 100 μl of the denatured solution at 45℃ overnight

[0105]

The monolayer of cells after hybridization was washed with the solution A for five minutes at 45℃, then washed with the solution B (10 M Tris HCl(pH8.5),500 mM NaCl) twice (5 minutes/ time), and treated with 20 μ□g/ml of RNase A/ solution B (pretreated at 90℃ for 10) at 37℃ for 20 minutes. They were washed with the solutions A and C with the order from solution A to solution C (50% of deionized formamide/1×SSC) at 45℃ for 30 minutes in each. In addition, they were washed with the solution C at room temperature for 20 minutes. Further, after they were washed with Buffer 1 (100 mM maleic acid, 150 M NaCl(pH7,5))(5 minutes × 2 times), treated with Buffer 2 (1% Blocking Reagent (Boehringer MannheimInc.)in Buffer I) at room temperature for 20 minutes.

[0106]

After the monolayer of cells were washed with Buffer I twice, FITC labeled anti-DIG antibodies (Fab, diluted with Buffer 2 by 100 times, protein level:1 μg/ml) was added, incubated for 30 minutes or more, washed with PBS(-) three times, and observed by fluorescence microscope; and the ratio of cells having IL-2 mRNA to the cells in the entire visual field was determined.

[0107]

Fig.37 and 38 show fluorescence micrographs when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 100 to 0. Fig.37 shows micrographs before flow cytometry. Fig.38 shows micrographs after flow cytometry. Fig.39 shows fluorescence micrographs

before flow cytometry when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 0 to 100. Fig.40 and 41 shows fluorescence micrographs when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 50 to 50. Fig.40 shows micrographs before flow cytometry. Fig.41 shows micrographs after flow cytometry. Fig.42 and 43 shows fluorescence micrographs when the mixing ratio of IL-2 expression-induced cells to -uninduced cell was 20 to 80. Fig.42 shows fluorescence micrographs before flow cytometer; and Fig.43 shows those after flow cytometry.

[0108]

The figures in the drawings represent the numbers of fluorescing cells per cells in the entire visual field. In Fig.37, 48/48 represents that all of the 48 cells were fluorescing cells in the image, suggesting that all the cells were IL-2 expressing cells. On the other hand, 0/32 in Fig.39 represents that there were no IL-2 expressing cells out of 32 cells. In Fig.40, 18/35 represents that 18 cells out of 35 cells were IL-2 expressing cells. In Fig.42, 8/39 represents 8 cells out of 39 cells were IL-2 expressing cells. These results are well consistent with the fact that IL-2 expression-induced and -uninduced cells were mixed with the ratios of 50 to 50 in Fig. 40 and 20 to 80 in Fig. 42. On the other hand, the figures in Fig.38, Fig.41, and Fig.43 were 9/9, 7/7, and 8/8, respectively, suggesting that IL-2 expressing cells were condensed from 20-50% to 100% throughout flow cytometry.

[0109]

<u>(14) Separation Method utilizing the Difference of Fluorescence Intensities of Live Cells which express Specific Genes</u>

Table 4 is a summarized result of (11)-(13) to show the effect of this separation method utilizing the differenciated intensities based on the fluorescence-labeled cellular IL-2 mRNA. The cells expressing IL-2 genes which were condensed from 20-50% to 100% by the separation methods utilizing the difference of fluorescence intensities. That is, the live cells expressing IL-2 genes were selectively separated from live cell groups consisting of IL-2 expressing and unexpressing cells at 100 % of purity.

[0110]

(Table 4)

Mixing Ratios		Flow Cytometry			
		Before		After	
IL-2 expression		FRET (D/A) Positive Cells(%)	Cells carrying IL-2 mRNA (%)	FRET (D/A) Positive Cells(%)	Cells carrying IL-2 mRNA (%)
Induced	uninduced				
100	0	20	100	100	100
0	100	0	0	-	-
50	50	5	51.4	100	100
20	80	2.8	20.5	100	100

[0111]

As described above, according to the present invention, it is possible to provide the separation method which allows to selectively separate and obtain the objective cells, that is, the cells which have expressed specific genes (which remain alive) when there are not cell surface molecules usable as markers in the cell, when cells cannot distinguished from each other though the markers exist, or when molecules to become markers were liberated to the extracellular liquid.

[0112]

SEQUENCE LISTING

<110> Laboratory of Molecular Biophotonics

<120> Method for selectively separating live cells that have expressed a specific gene

<130> P99MB-016

<140>

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<160> 10

<170> PatentIn Ver. 2.1

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[BRIEF DESCRIPTION OF DRAWINGS]

[FIG. 1]

Fig.1 is a drawing of the entire base sequence of the IL-2 mRNA and the base sequence of the oligo DNA probe.

[FIG. 2]

Fig.2 is a drawing of a fluorescence spectrum of a hybrid which the donor probe 228-242(D) and the acceptor probe 243-257(A) were adjacent to and hybridized with IL-2 RNA.

[FIG. 3]

Fig.3 is a drawing of a fluorescence spectrum of a hybrid which the donor probe 198-212(D) and the acceptor probe 213-227(A) were adjacent to and hybridized with IL-2 RNA.

[FIG. 4]

Fig.4 is a drawing of a fluorescence spectrum of a hybrid which the donor probe 77-91(D) and the acceptor probe 92-107(A) were adjacent to and hybridized with IL-2 RNA.

[FIG. 5]

Fig.5 is a drawing of a fluorescence spectrum of a hybrid which the donor probe 287-91(D) and the acceptor probe 302-316(A) were adjacent to and hybridized with IL-2 RNA.

[FIG. 6]

Fig.6 is a drawing of a fluorescence spectrum of a hybrid which the donor probe 342-356(D) and the acceptor probe 357-301(A) were adjacent to and hybridized with IL-2 RNA.

[FIG. 7]

Fig.7 is a drawing of a HPLC chromatogram obtained when the mixture solution of the donor probe 342-356(D) and IL-2 RNA was separated with HPLC.

[FIG. 8]

Fig.8 is a drawing of a HPLC chromatogram obtained when the mixture solution of the acceptor probe 357-371(D) and IL-2 RNA was separated with HPLC.

[FIG. 9]

Fig.9 is a drawing of the amount of IL-2 secreted by Jurkat E6-1 cells associated with the expression induction treatment of IL-2, the fluorescence microphotographs which IL-2 mRNA in the extract from the cells were fluorescence-detected, and the number of molecules of the intracellular IL-2 mRNA.

[FIG. 10]

Fig.10 is a drawing of the fluorescence microphotographs to which DIG labeled dUTP and reverse transcriptase were added, oligonucleotide was not introduced, and all of them were not introduced or added, after oligonucleotide (oligo dT or oligo dA) was introduced into the fixed state IL-2 expression-induced cells and IL-2 expression-uninduced cells.

[FIG. 11]

Fig.11 is a drawing of fluorescence microphotographs which

hybrids were detected and formed between IL-2 mRNA in the fixed state IL-2 expression-induced cells and IL-2 expression-uninduced cells and various probes (without fluorescent marker).

[FIG. 12]

Fig.12 is a drawing showing the result which hybrids formed between the IL-2 mRNA in the fixed state IL-2 expression-induced cells and IL-2 expression-uninduced cells and various probes (without fluorescent marker) were fluorescence-detected, then the fluorescence intensity was standardized based on the values of fluorescence intensity generated from the composites of fluorescent labeled compounds formed with total mRNA in said cells and oligo dT.

[FIG. 13]

Fig.13 is a drawing of fluorescence microphotographs of D/A images, D/D images and A/A images at the state of excitation of donor fluorescent dyes of hybrids formed with IL-2 mRNA in the fixed state IL-2 expression-induced cells and IL-2 expression-uninduced cell, each donor probe and acceptor probe.

[FIG. 14]

Fig.14 is a drawing of the result of the fluorescence intensity values of D/A representing the hybrids, standardized based on the intensity values of A/A representing all the acceptor probes in said cells, by fluorescence-measuring of the acceptor fluorescence intensity when the donor fluorescent dyes of the hybrids, which were formed with IL-2 mRNA in the fixed state IL-2 expression-induced cells and IL-2 expression-uninduced cells and each donor probe and acceptor probe.

[FIG. 15]

Fig.15 is a drawing of the fluorescence micrographs of D/A, D/D, A/A images, and the corresponding phase contrast micrograph of the living IL-2 expression-induced and -uninduced cells.

[FIG. 16]

Fig.16 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 100:0 was subjected to flow cytometry and dot-plotted based on forward scattering light and side scattering light (R1 is the region selected as typical live cells to be measured.)

[FIG. 17]

Fig.17 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 100:0 was subjected to flow cytometry and dot-plotted based on the relative fluorescence intensity by energy donor fluorescent dye and the relative fluorescence of energy acceptor fluorescent dyes according to FRET (R2 is the region selected as fluorescing cells according to the FRET.)

[FIG. 18]

Fig.18 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 0:100 was subjected to flow cytometry and dot-plotted based on forward scattering light and side scattering light.

[FIG. 19]

Fig.19 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 0:100 was subjected to flow cytometry and dot-plotted based on the relative fluorescence intensity by energy donor fluorescent dyes and the relative fluorescent intensity of

energy acceptor fluorescent dyes according to FRET.

[FIG. 20]

Fig.20 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 50:50 was subjected to flow cytometry and dot-plotted based on forward scattering light and side scattering light (R1 is the region selected as typical live cells to be measured.)

[FIG. 21]

Fig.21 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 50:50 was subjected to flow cytometry and dot-plotted based on the relative fluorescence intensity by energy donor fluorescent dye and the relative fluorescence of energy acceptor fluorescent dyes according to FRET (R2 is the region selected as fluorescing cells according to the FRET.)

[FIG. 22]

Fig.22 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 20:80 was subjected to flow cytometry and dot-plotted based on forward scattering light and side scattering light (R1 is the region selected as typical live cells to be measured.)

[FIG. 23]

Fig.23 is a drawing which the mixture of IL-2 expression-induced cells and -uninduced cells with the ratio of 80:20 was subjected to flow cytometry and dot-plotted based on the relative fluorescence intensity by energy donor fluorescent dyes and the relative fluorescent intensity of energy acceptor fluorescent dyes according to FRET.

[FIG. 24]

Fig.24 is a drawing which the selectively separated cells suitable for both R1 in Fig.16 and R2 in Fig.17 were subjected to flow cytometry and dot-plotted based on forward scattering light and side scattering light.

[FIG. 25]

Fig.25 is a drawing which the selectively separated cells suitable for both R1 in Fig.16 and R2 in Fig.17 were subjected to flow cytometry and dot-plotted based on the relative fluorescence intensity by energy donor fluorescent dyes and the relative fluorescent intensity of energy acceptor fluorescent dyes according to FRET.

[FIG. 26]

Fig.26 is a drawing which the selectively separated cells suitable for both R1 in Fig.20 and R2 in Fig.21 were subjected to flow cytometry and dot-plotted based on forward scattering light and side scattering light.

[FIG. 27]

Fig.27 is a drawing which the selectively separated cells suitable for both R1 in Fig.20 and R2 in Fig.21 were subjected to flow cytometry and dot-plotted based on the relative fluorescence intensity by energy donor fluorescent dyes and the relative fluorescent intensity of energy acceptor fluorescent dyes according to FRET.

[FIG. 28]

Fig.28 is a drawing which the selectively separated cells suitable for both R1 in Fig.22 and R2 in Fig.23 were subjected to flow cytometry and dot-plotted based on forward scattering light and side scattering light.

[FIG. 29]

Fig.29 is a drawing which the selectively separated cells suitable for

both R1 in Fig.22 and R2 in Fig.23 were subjected to flow cytometry and dot-plotted based on the relative fluorescence intensity by energy donor fluorescent dyes and the relative fluorescent intensity of energy acceptor fluorescent dyes according to FRET.

[FIG. 30]

Fig.30 is a drawing which consists of D/A, D/D, A/A image, and the corresponding phase contrast micrograph of the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 100:0 before flow cytometry.

[FIG. 31]

Fig.31 is a drawing which consists of D/A, D/D, A/A image, and the corresponding phase contrast micrograph of the selectively separated cells suitable for both R1 in Fig.16 and R2 in Fig.17.

[FIG. 32]

Fig.32 is a drawing which consists of D/A, D/D, A/A image, and the corresponding phase contrast micrograph of the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 50:50 before flow cytometry.

[FIG. 33]

Fig.33 is a drawing which consists of D/A, D/D, A/A image, and the corresponding phase contrast micrograph of the selectively separated cells suitable for both R1 in Fig.20 and R2 in Fig.21.

[FIG. 34]

Fig.34 is a drawing which consists of D/A, D/D, A/A image, and the corresponding phase contrast micrograph of the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 20:80 before

flow cytometry.

[FIG. 35]

Fig.35 is a drawing which consists of D/A, D/D, A/A image, and the corresponding phase contrast micrograph of the selectively separated cells suitable for both R1 in Fig.22 and R2 in Fig.23.

[FIG. 36]

Fig.36 is a drawing which consists of D/A, D/D, A/A image, and the corresponding phase contrast micrograph of the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 0:100.

[FIG. 37]

Fig.37 is a drawing of fluorescence micrograph which the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 100:0 before flow cytometry was fixed to a glass-bottomed dish to detect a hybrid formed between cellular IL-2 mRNA and a RNA probe complementary to IL-2 mRNA.

[FIG. 38]

Fig.38 is a drawing of fluorescence micrograph which the selectively separated cells suitable for both R1 in Fig.16 and R2 in Fig. 17, were fixed to a glass-bottomed dish to detect a hybrid formed between cellular IL-2 mRNA and a RNA probe complementary to IL-2 mRNA.

[FIG. 39]

Fig.39 is a drawing of fluorescence micrograph which the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 0:100 was fixed to a glass-bottomed dish to detect a hybrid formed between cellular IL-2 mRNA and a RNA probe complementary to IL-2 mRNA.

[FIG. 40]

Fig.40 is a drawing of fluorescence micrograph which the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 50:50 was fixed to a glass-bottomed dish to detect a hybrid formed between cellular IL-2 mRNA and a RNA probe complementary to IL-2 mRNA.

[FIG. 41]

Fig.41 is a drawing of fluorescence micrograph which the selectively separated cells suitable for both R1 in Fig.20 and R2 in Fig. 21, were fixed to a glass-bottomed dish to detect a hybrid formed between cellular IL-2 mRNA and a RNA probe complementary to IL-2 mRNA.

[FIG. 42]

Fig.42 is a drawing of fluorescence micrograph which the mixture of living IL-2 expression-induced and -uninduced cells with the ratio of 20:80 was fixed to a glass-bottomed dish to detect a hybrid formed between cellular IL-2 mRNA and a RNA probe complementary to IL-2 mRNA.

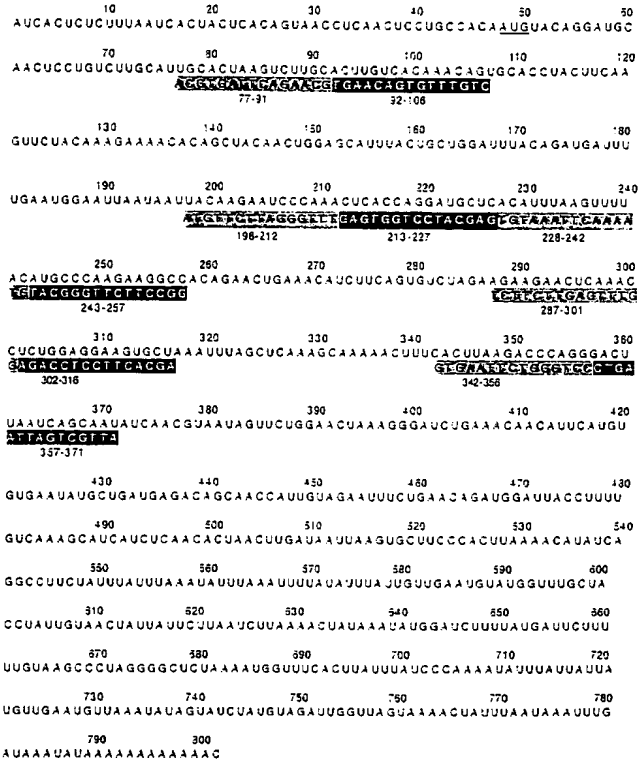
[FIG. 43]

Fig.43 is a drawing of fluorescence micrograph which the selectively separated cells suitable for both R1 in Fig.22 and R2 in Fig. 23, were fixed to a glass-bottomed dish to detect a hybrid formed between cellular IL-2 mRNA and a RNA probe complementary to IL-2 mRNA.

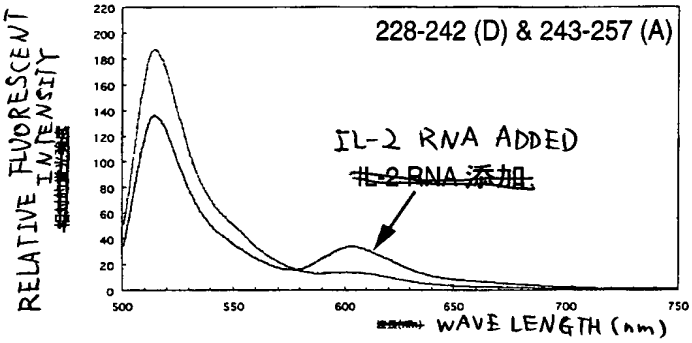
[FIG. 44]

Fig.44 is a drawing which indicates the correlation in cell differentiation of various cells forming the organic immune system and which also indicates that various cells cooperate with and check each other through cytokines such as interleukin (IL), and the immunological functions in organism is kept constant.

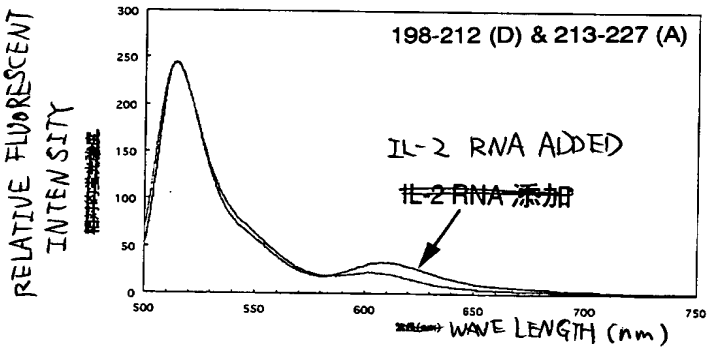
【Fig.1】



【Fig.2】



【Fig.3】



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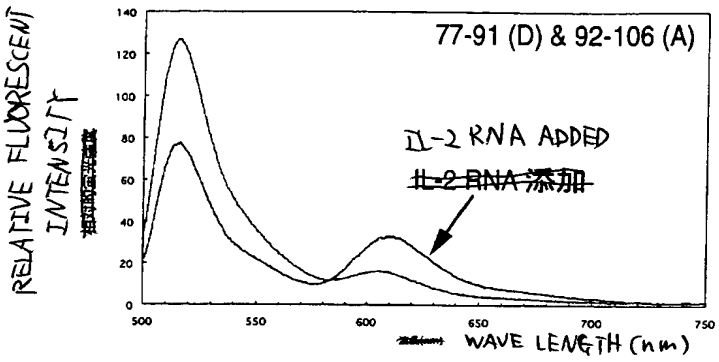
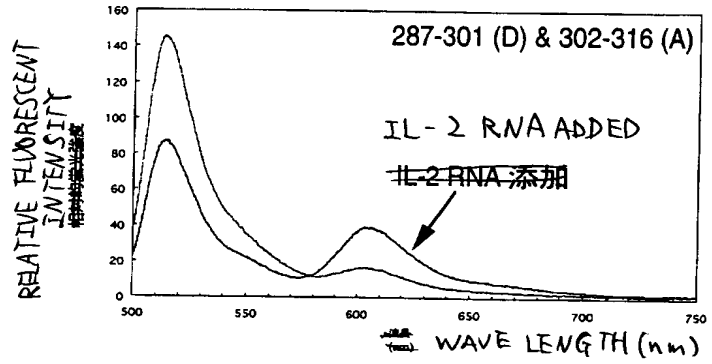


【Fig.5】



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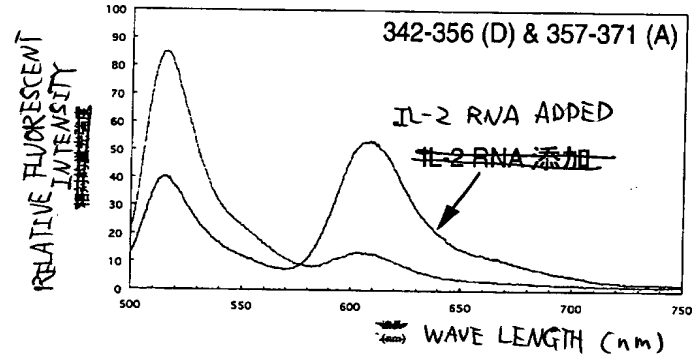
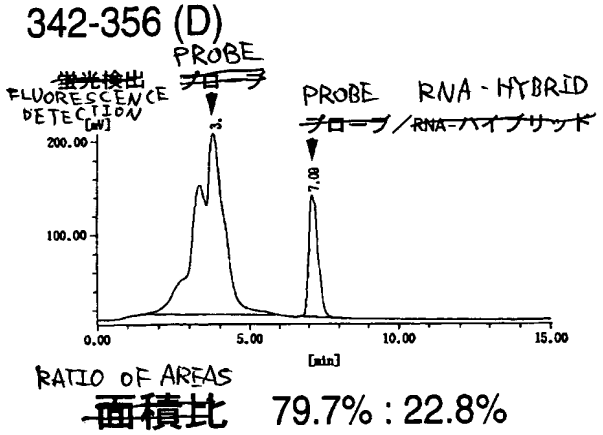


【Fig.7】



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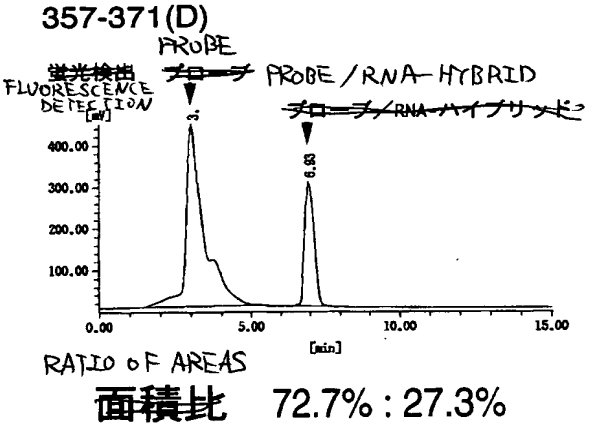
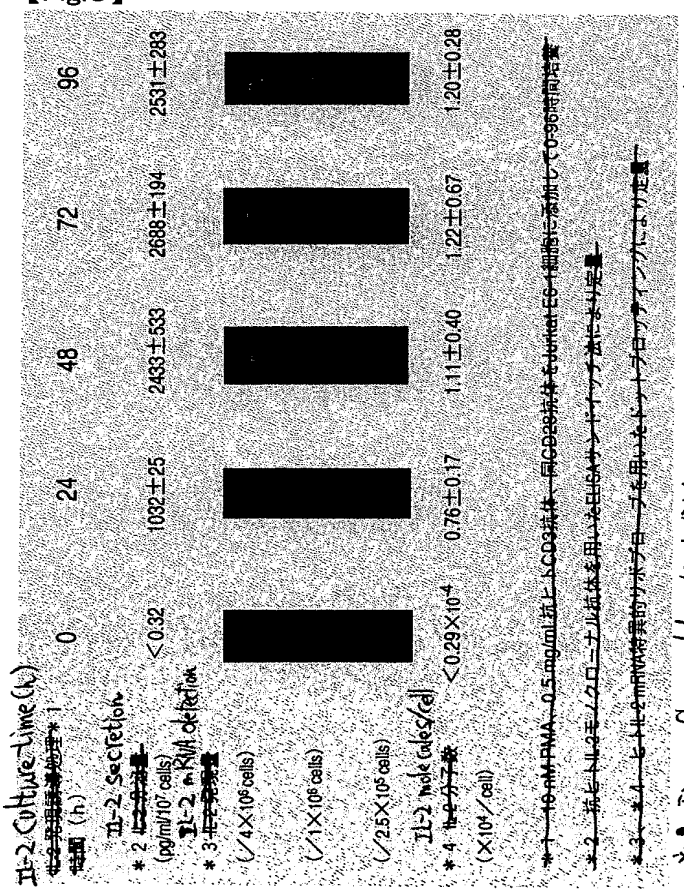


【Fig.9】

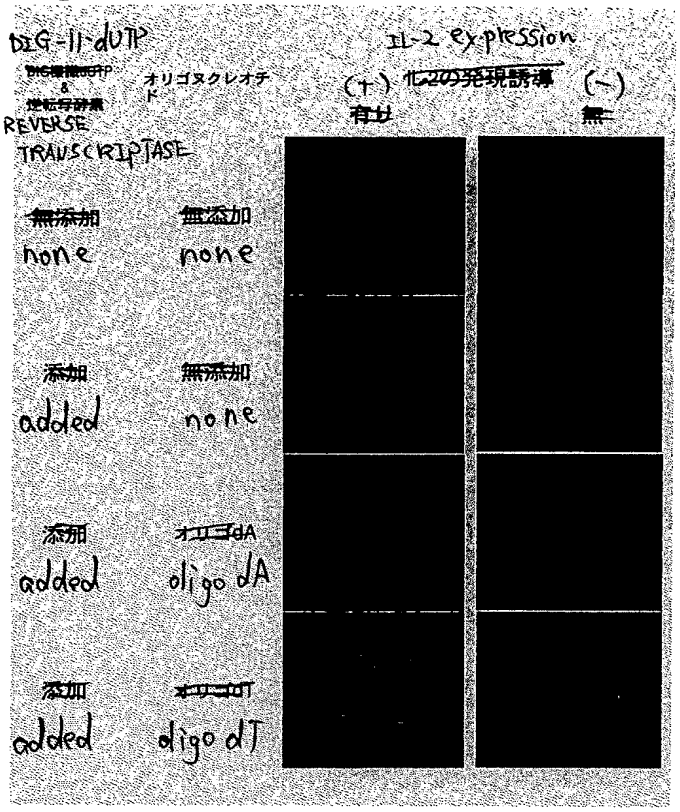


*1 Time after adding 10nM PMA, 0.5mg/ml anti-human CD3 antibody and antihuman CD28 antibody to cultured Jurkat E6-1 cells

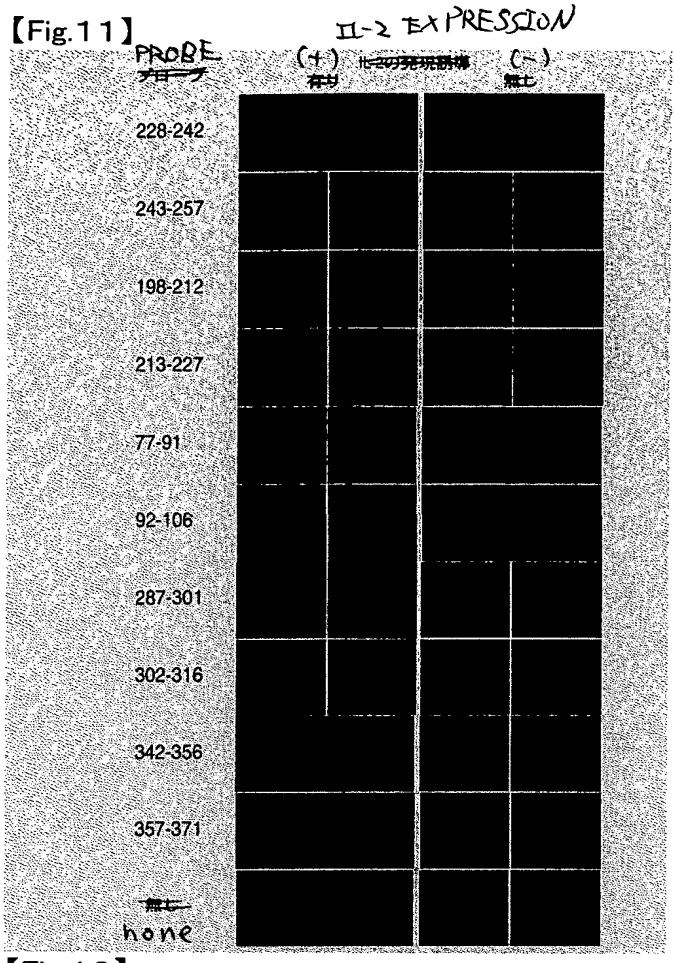
*2 The concentration of IL-2 in the culture supernatants measured by ELISA using anti-human IL-2 antibody

*3,4 Determination of cellular IL-2 expression level by dot blotting using RNA probe complementary to human IL-2 mRNA

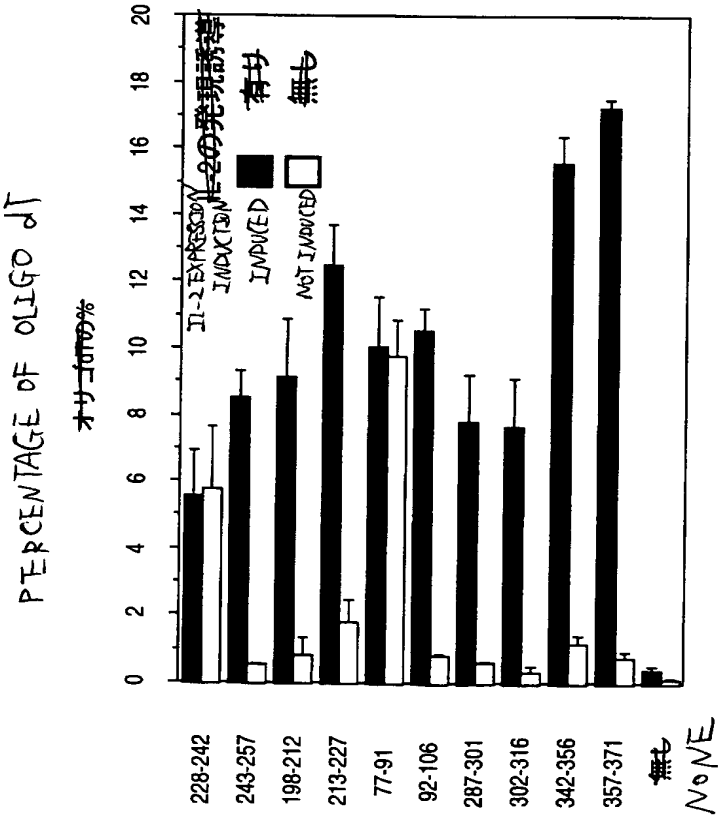
【Fig.10】


DIG-11-dUTP
DIG標識dUTP & 逆転写酵素 REVERSE TRANSCRIPTASE
オリゴヌクレオチド
IL-2 expression
(+) 有り
IL-2の発現誘導
(-) 無し
無添加 none
無添加 none
添加 added
無添加 none
添加 added
オリゴdA oligo dA
添加 added
オリゴdT oligo dT

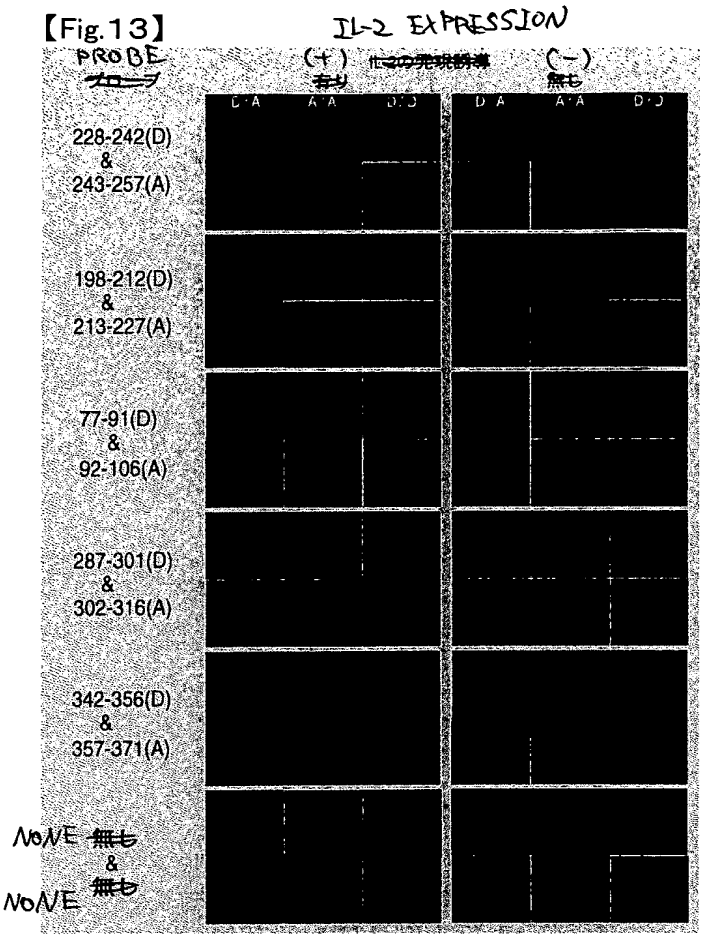
【Fig.11】


IL-2 EXPRESSION
PROBE
プローブ
(+)
有り
(−)
無し
228-242
243-257
198-212
213-227
77-91
92-106
287-301
302-316
342-356
357-371
無し
none

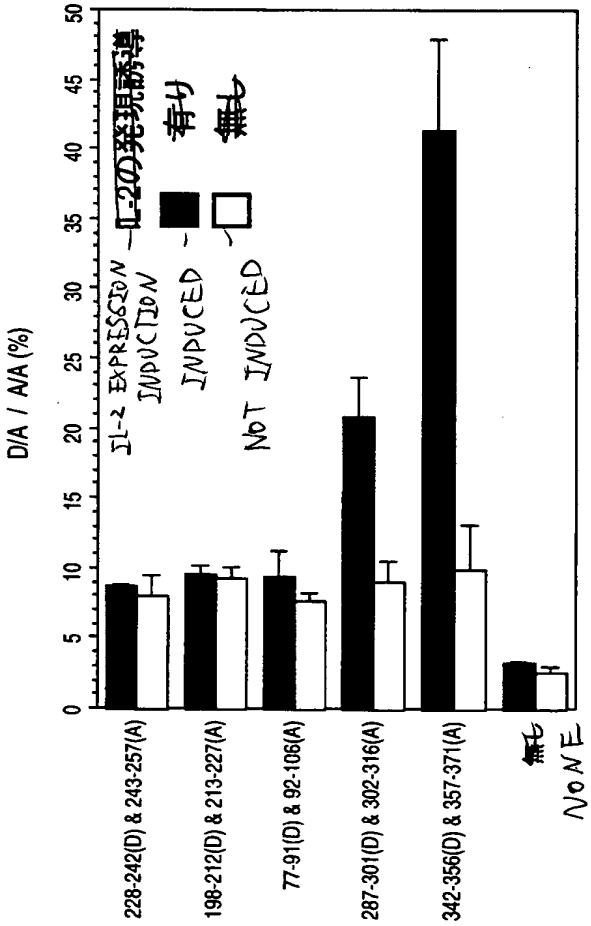
【Fig.12】


PERCENTAGE OF OLIGO dT
IL-2 EXPRESSION
INDUCED
NOT INDUCED
有り
無し
0
2
4
6
8
10
12
14
16
18
20
228-242
243-257
198-212
213-227
77-91
92-106
287-301
302-316
342-356
357-371
無し
NONE

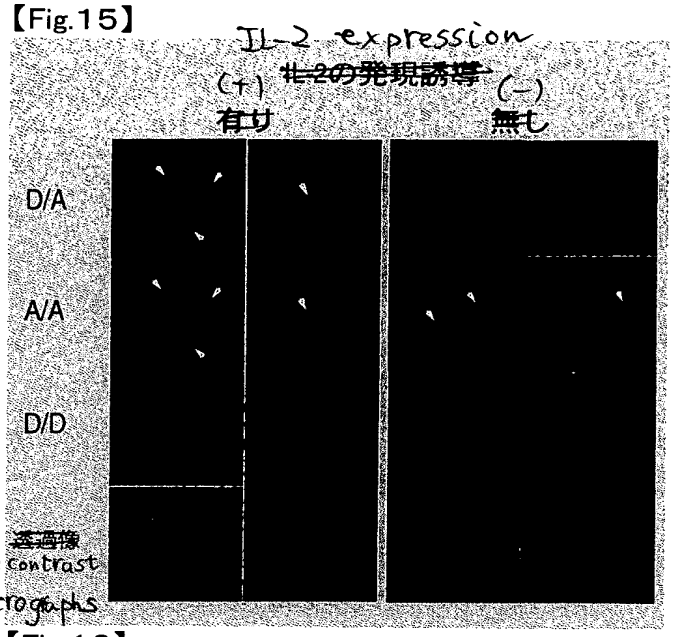
【Fig.13】


IL-2 EXPRESSION
PROBE
プローブ
(+)
有り
(−)
無し
D-A
A-A
D-D
228-242(D)
&
243-257(A)
198-212(D)
&
213-227(A)
77-91(D)
&
92-106(A)
287-301(D)
&
302-316(A)
342-356(D)
&
357-371(A)
NoNE 無し
&
NoNE 無し

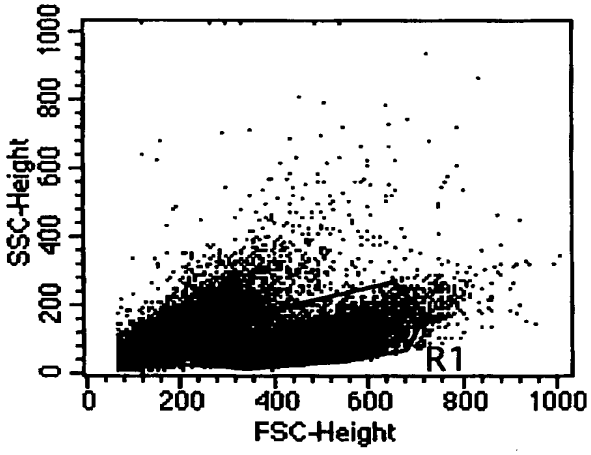
【Fig.14】


D/A / A/A (%)
0
5
10
15
20
25
30
35
40
45
50
IL-2 EXPRESSION INDUCTION
IL-2の発現誘導
INDUCED
有り
NOT INDUCED
無し
228-242(D) & 243-257(A)
198-212(D) & 213-227(A)
77-91(D) & 92-106(A)
287-301(D) & 302-316(A)
342-356(D) & 357-371(A)
無し
NoNE

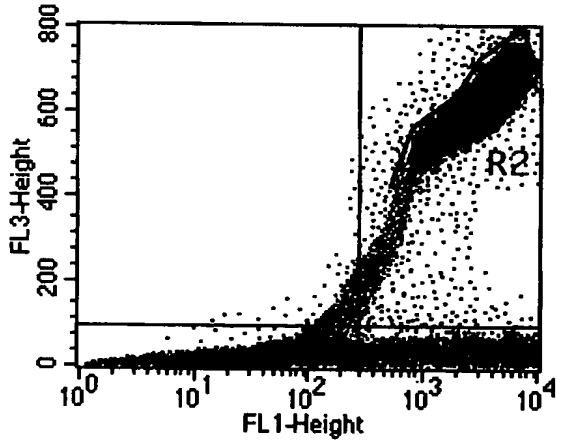
【Fig.15】



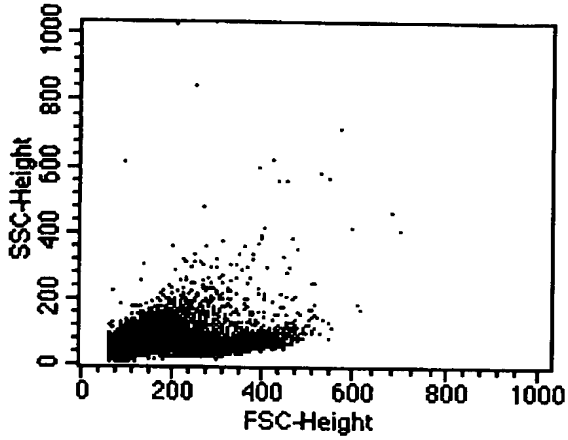
【Fig.16】



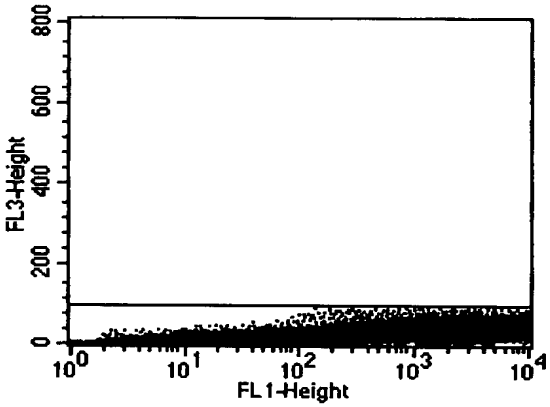
【Fig.17】



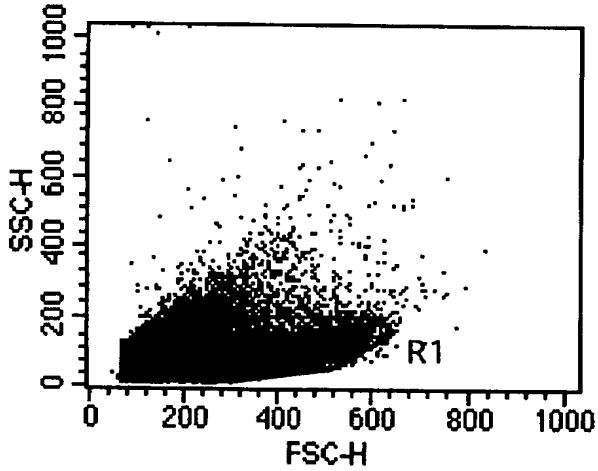
【Fig.18】



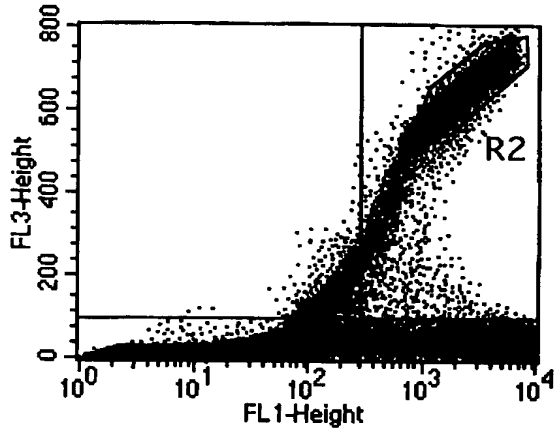
【Fig.19】



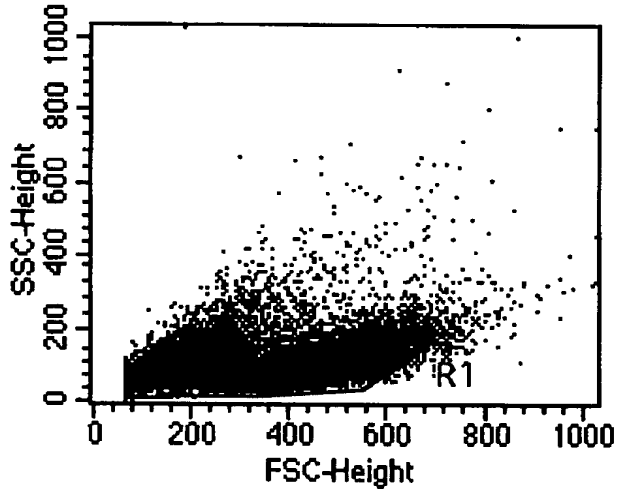
【Fig.20】



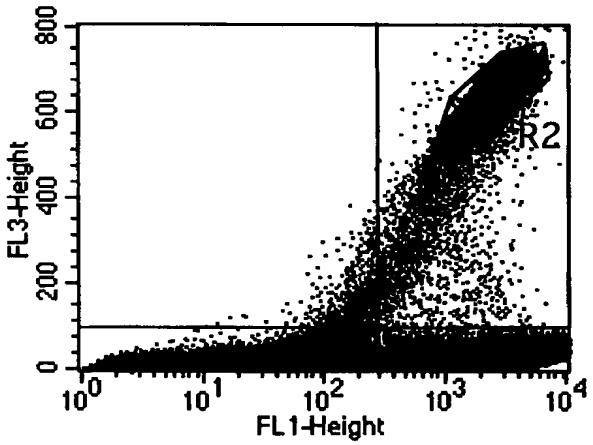
【Fig.21】



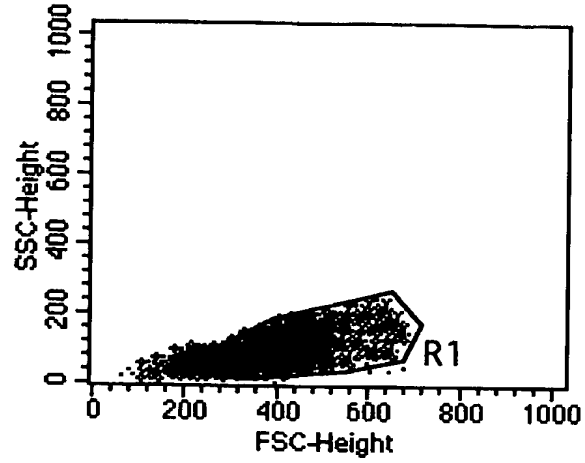
【Fig.22】



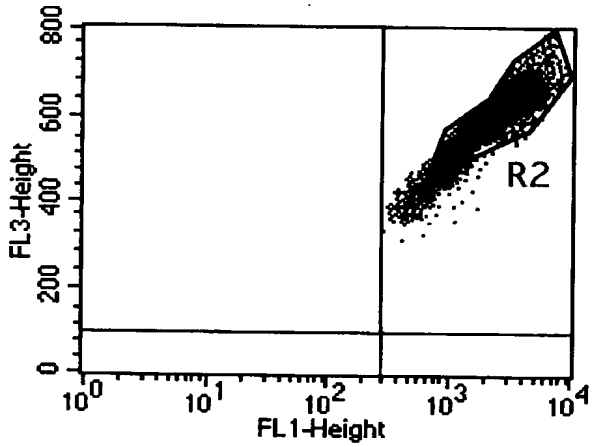
【Fig.23】



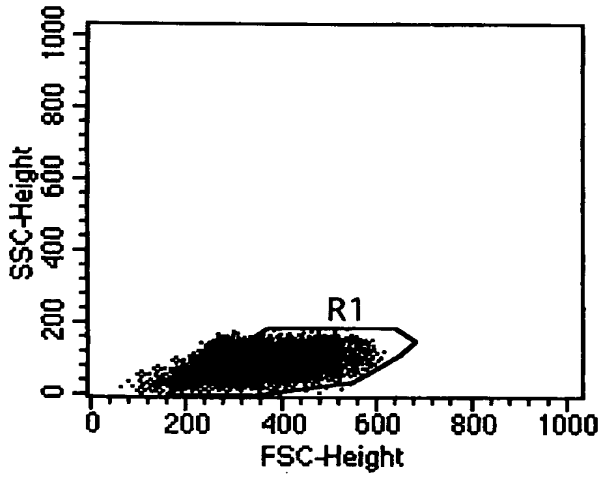
【Fig.24】



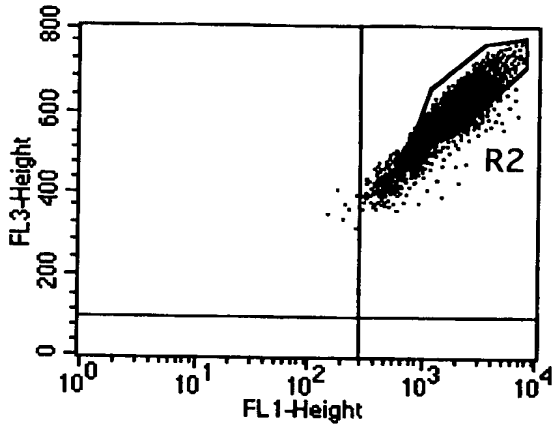
【Fig.25】



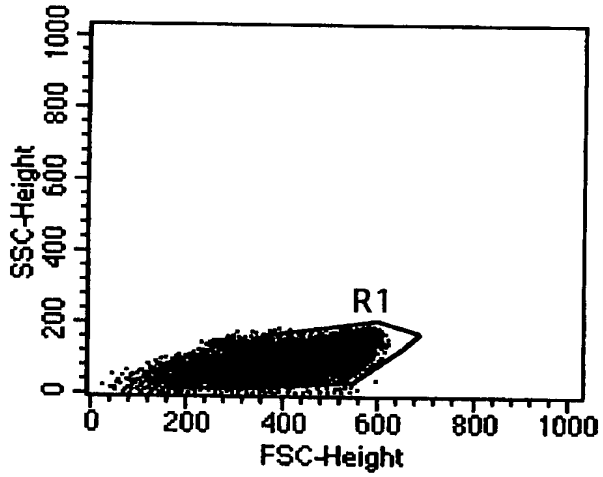
【Fig.26】



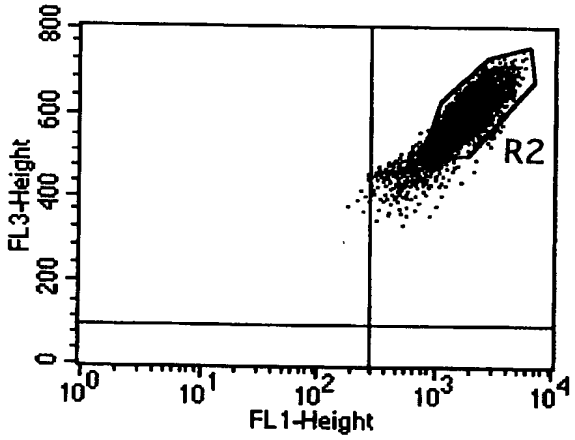
【Fig.27】



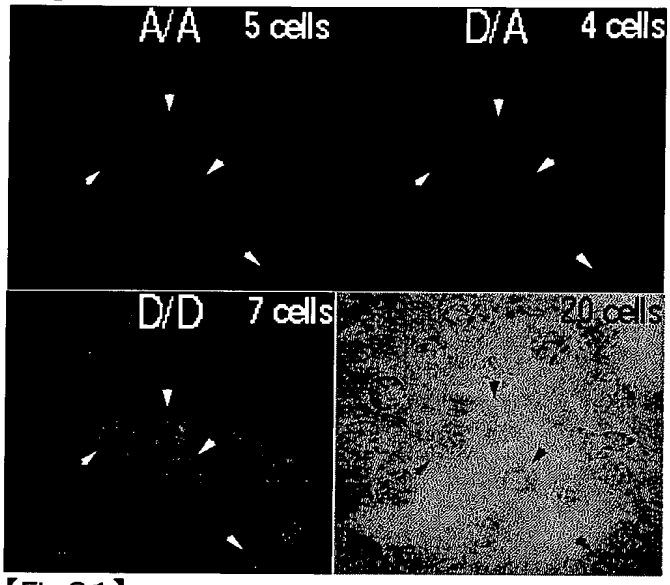
【Fig.28】



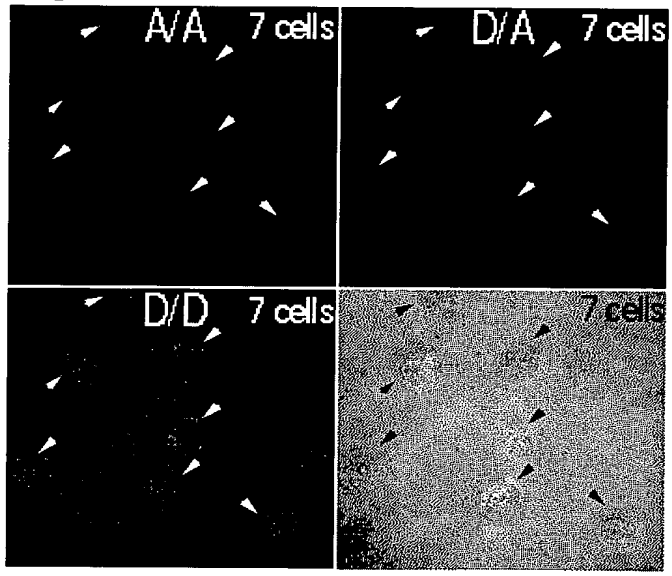
【Fig.29】



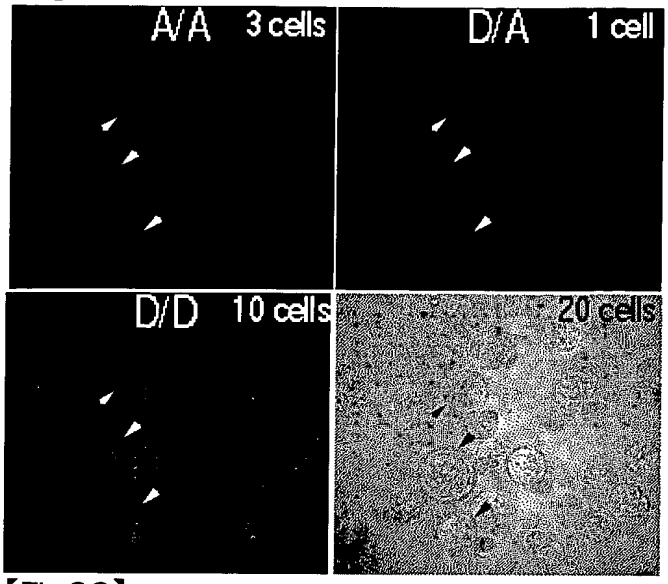
【Fig.30】


A/A 5 cells
D/A 4 cells
D/D 7 cells
20 cells

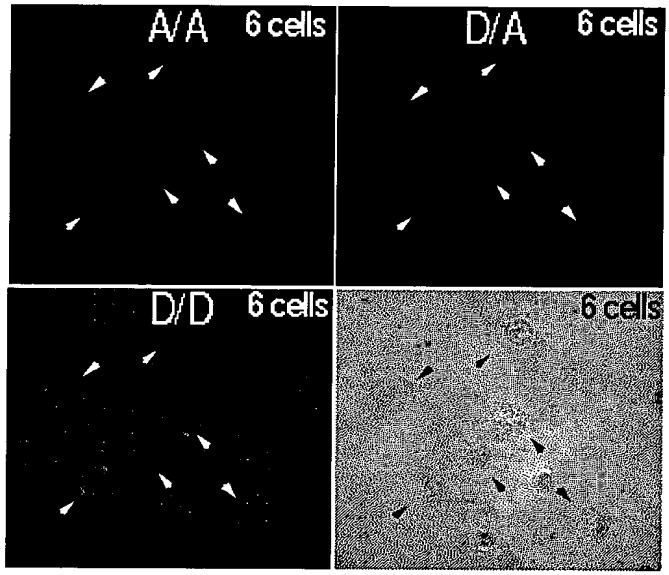
【Fig.31】


A/A 7 cells
D/A 7 cells
D/D 7 cells
7 cells

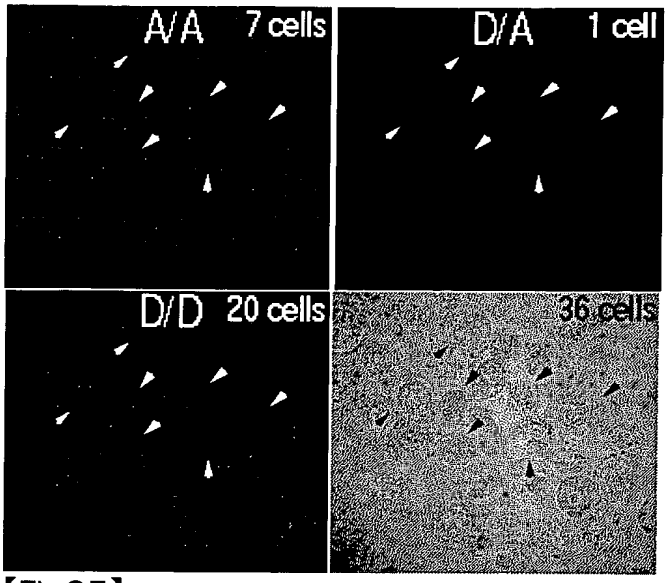
【Fig.32】


A/A 3 cells
D/A 1 cell
D/D 10 cells
20 cells

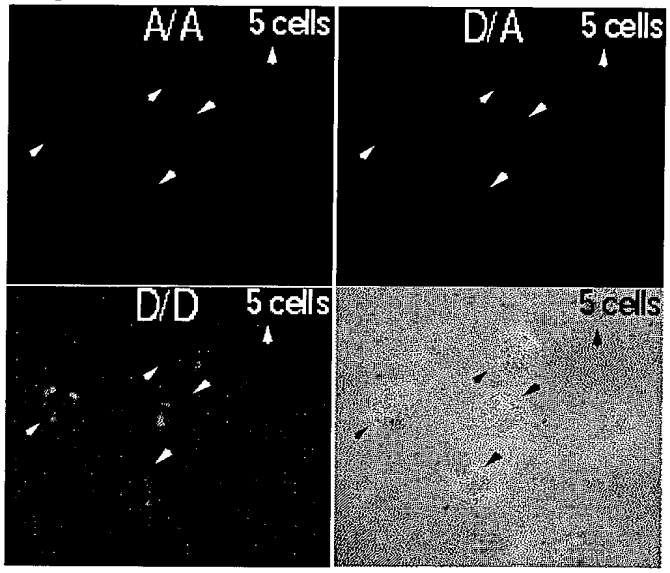
【Fig.33】


A/A 6 cells
D/A 6 cells
D/D 6 cells
6 cells

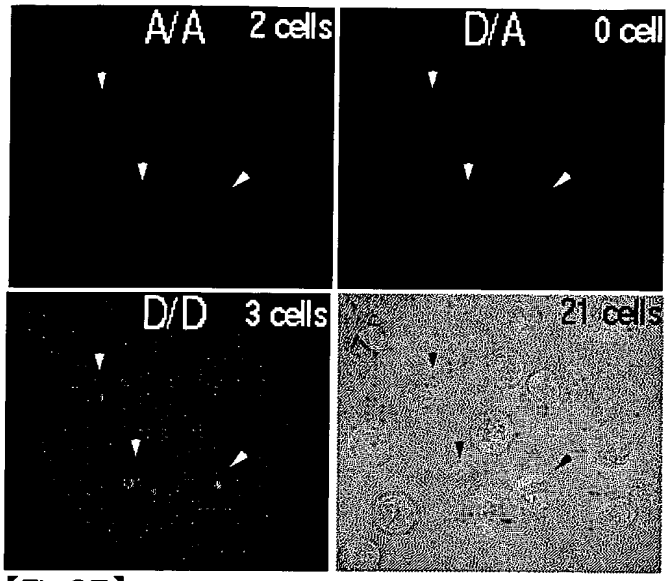
【Fig.34】


A/A 7 cells
D/A 1 cell
D/D 20 cells
36 cells

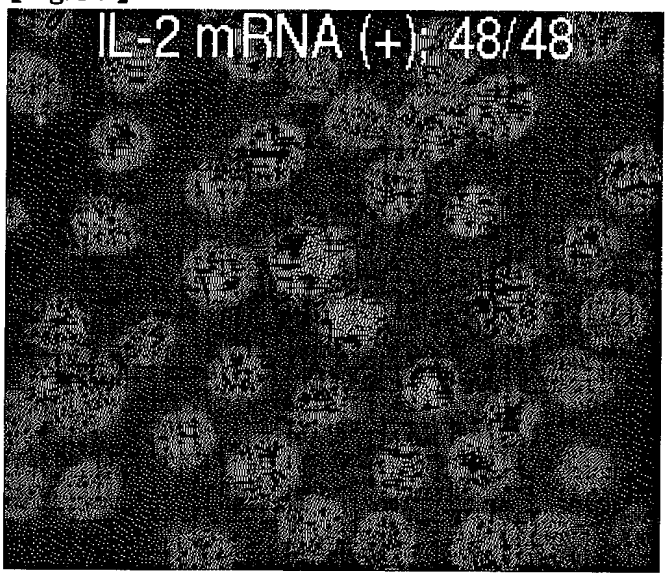
【Fig.35】


A/A 5 cells
D/A 5 cells
D/D 5 cells
5 cells

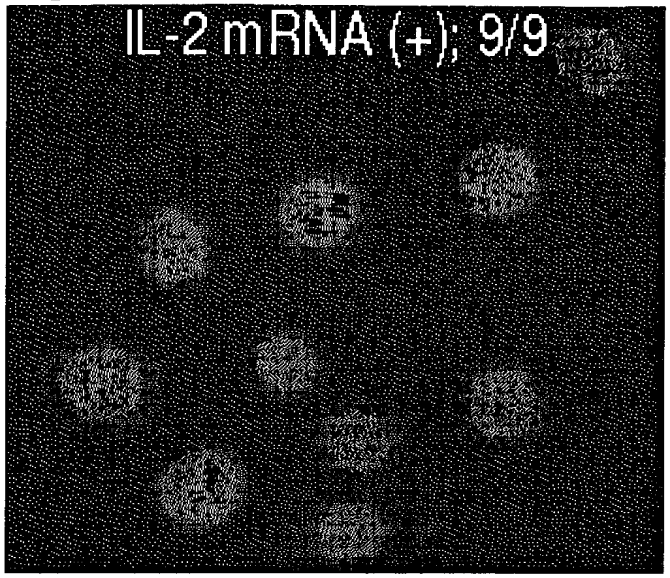
【Fig.36】



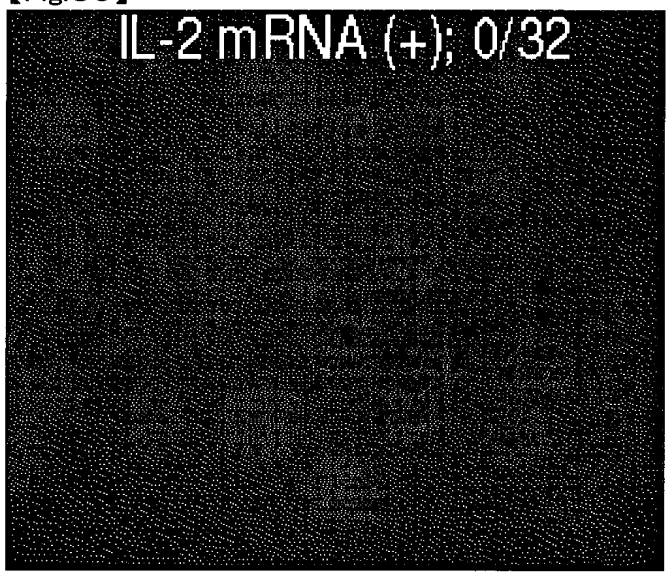
【Fig.37】



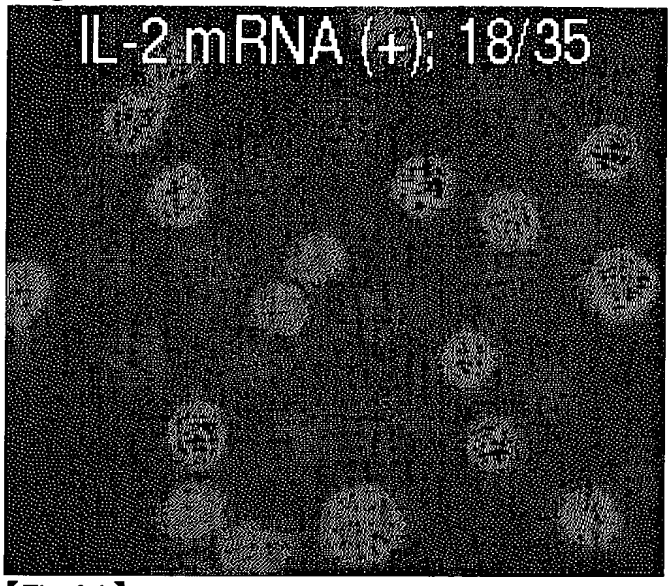
【Fig.38】



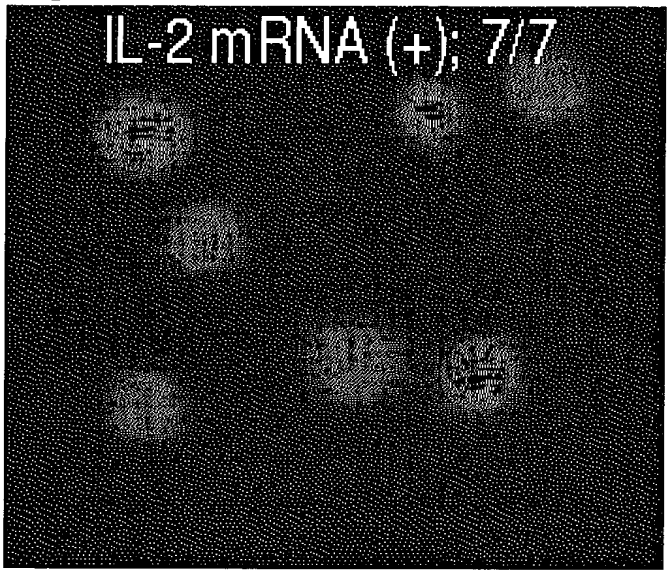
【Fig.39】



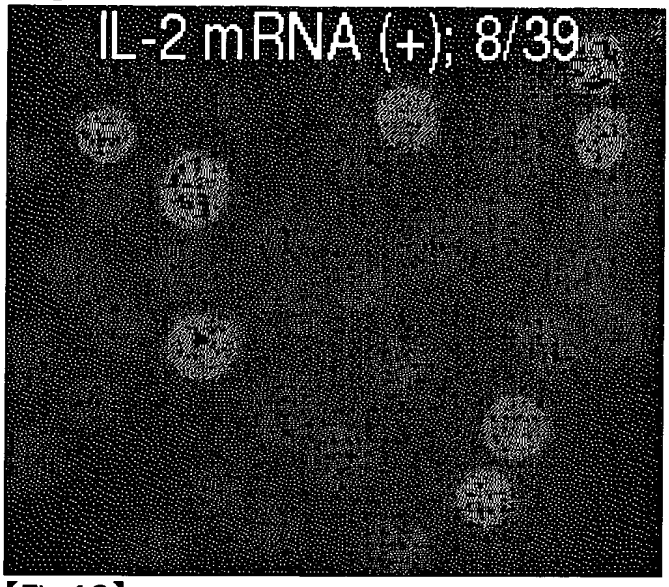
【Fig.40】



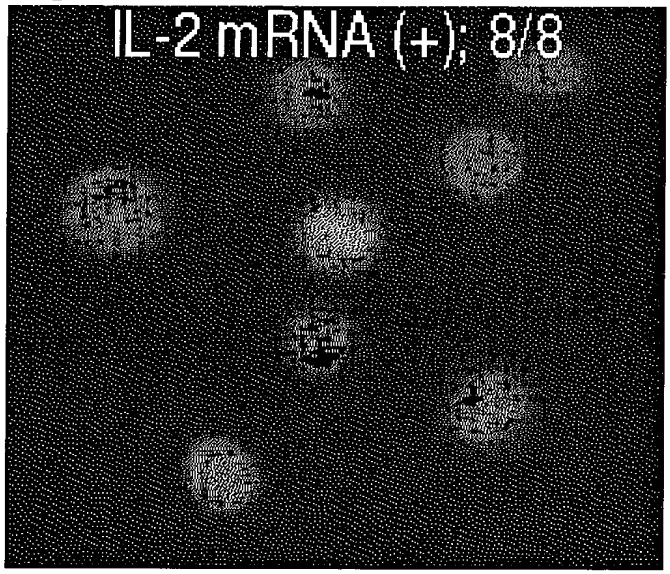
【Fig.41】



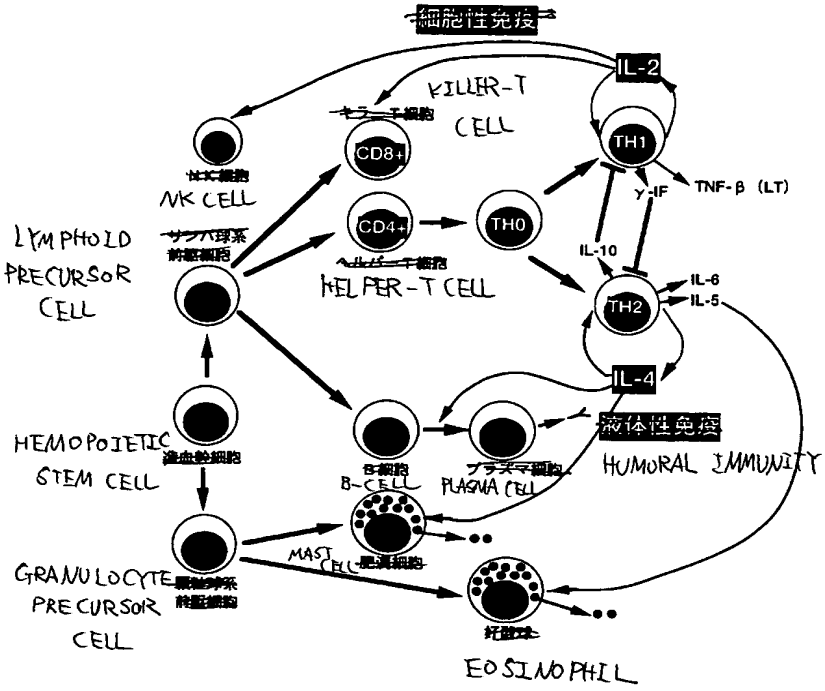
【Fig.42】



【Fig.43】



【Fig.44】 CELLULAR IMMUNITY



[NAME OF DOCUMENT] Abstract

[ABSTRACT]

[OBJECT] To provide the separation method which allows to selectively separate and obtain the objective cells, that is, the cells which have expressed specific genes, when there are not cell surface molecules usable as markers in the cell, when cells cannot distinguished from each other if present, or even when molecules to become markers are liberated to the extracellular liquid.

[SOLUTION] The present invention provides a method for selectively separating a live cell which has expressed a specific gene comprising a first step of introducing markers which label mRNA into cells in a live cell group containing live cells which have expressed a specific gene, a second step of labeling said mRNA with said marker to obtain live cell group containing live cells having the labeled mRNA, and a third step of detecting said labeled mRNA to identify the live cells having the labeled mRNA and separating the identified live cells selectively from said live cell group obtained in said second step.

[SELECTED DRAWING] none

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(54) Nucleic acid detection in cytoplasm

(57) Detection probes labeled with fluorescent dyes, to which are bound nuclear membrane unpermeable molecules via linkers, having base sequences that can hybridize to a target nucleic acid. The probes are introduced into the cytoplasm of a living cell in which the target nucleic acid is present, and the target nucleic acid is detected by measurement of the change in fluorescence of the fluorescent dyes due to the formation of a hybrid of the target nucleic acid and the probes.

Fig.1A

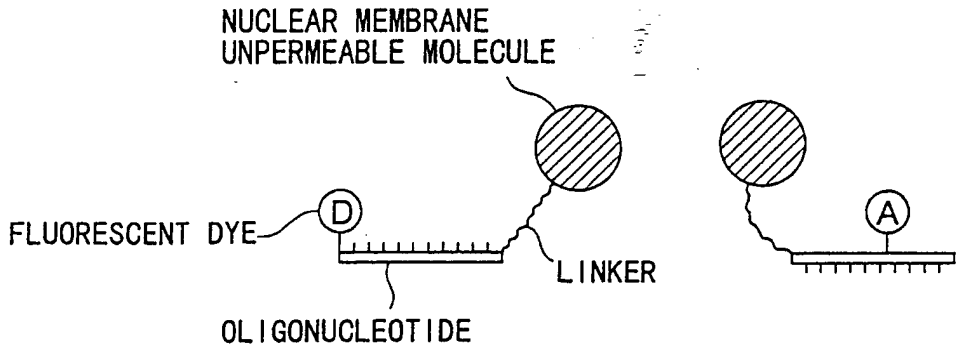

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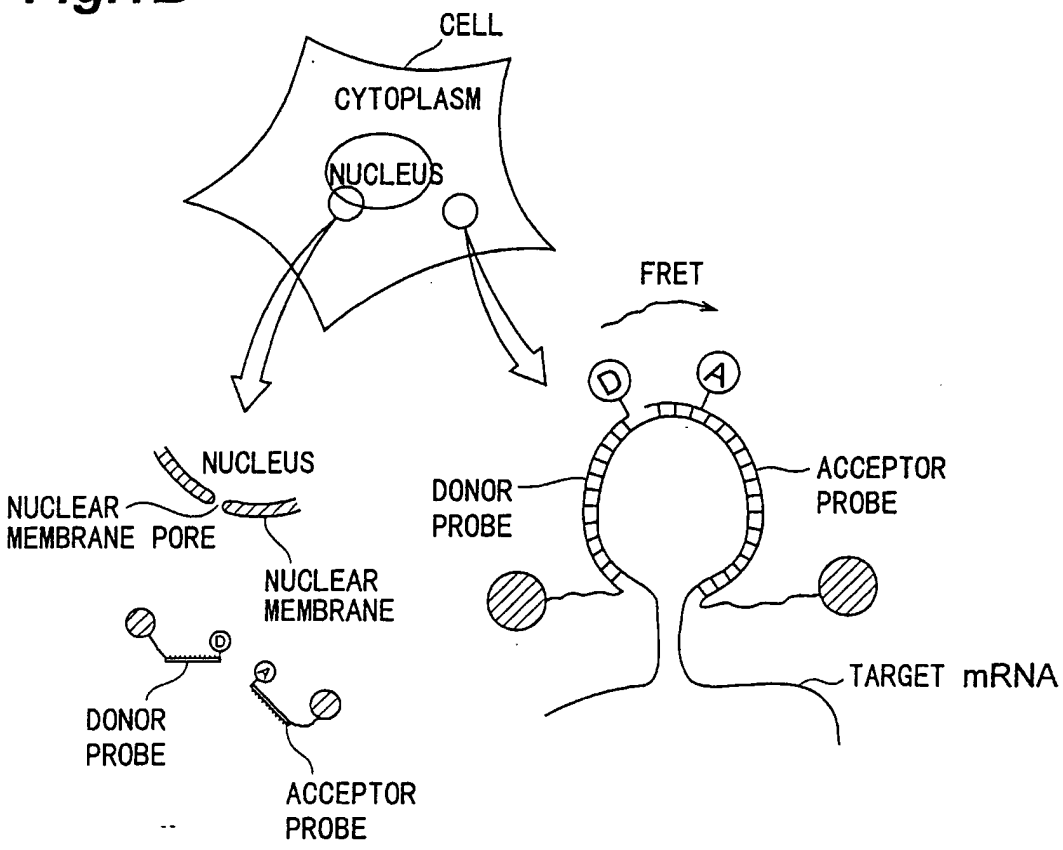
Fig.1B


CELL
CYTOPLASM
NUCLEUS
FRET
D
A
NUCLEUS
NUCLEAR MEMBRANE PORE
NUCLEAR MEMBRANE
DONOR PROBE
ACCEPTOR PROBE
DONOR PROBE
ACCEPTOR PROBE
TARGET mRNA

Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to a method for the detection of a target nucleic acid in the cytoplasm of a living cell. More specifically, it relates to a method for the detection of a target nucleic acid existing in the cytoplasm of a living cell through hybridization by using probes labeled with fluorescent dyes.

Related Background Art

[0002] Hybridization is known as one of the methods to detect a nucleic acid having a specified base sequence (hereafter referred to as "target nucleic acid"). This method employs an oligonucleotide probe having a base sequence capable of hybridizing to the target nucleic acid as a detection probe to form a hybrid, and performs detection of the target nucleic acid by detecting the hybrid through various detection means.

[0003] Hybridization methods in the prior art, however, suffer from the drawbacks described below. Thus it will be difficult to apply them to detecting a target nucleic acid in the cytoplasm of a living cell. In other words, when a detection probe is introduced into the cytoplasm, it will rapidly move to the nucleus. This makes it difficult to allow the probe to form a hybrid with the target nucleic acid existing in the cytoplasm. In addition, the detection probe, which has been introduced into the cytoplasm, or the hybrid between the detection probe and the target nucleic acid is rapidly digested by various kinds of nuclease existing in the cytoplasm, which renders the detection of the target nucleic acid difficult.

SUMMARY OF THE INVENTION

[0004] The present inventors have discovered that a detection probe having a specified structure does not rapidly move to nucleus when introduced into the cytoplasm of a living cell and that it is not easily digested by nuclease, and thus have accomplished this invention. Specifically, when the detection probe having the specified structure is introduced into the cytoplasm, the probe does not readily move into the nucleus; nor is it readily digested by the nuclease. The probe forms a hybrid with a target nucleic acid existing in the cytoplasm, and the hybrid will be detectable without being subjected to digestion by the nuclease. Accordingly, when such a detection probe is used, the method for the detection of a target nucleic acid existing in the cytoplasm of a living cell (hereafter referred to as "the detection method of this invention) will be attained.

[0005] Specifically, the detection probe to be used in the method of this invention has the following characteristics: it is an oligonucleotide probe having a base sequence capable of hybridizing to the specified base sequence of a target nucleic acid; it is provided with a molecule that prevents the movement of the oligonucleotide into nucleus through nuclear membrane pores, and preferably, blocks the digestion of the oliginucleotide by nuclease; and it is provided with a fluorescent label that only allows the detection of the hybrid with the target nucleic acid.

[0006] Preferably, the detection probe to be used in the detection method of this invention is a pair of probes comprising two types of oligonucleotide probe which are respectively bound to an energy donor fluorescent dye (also referred to as "donor fluorescent dye" or simply as "donor" hereafter) and an energy acceptor fluorescent dye (also referred to as "acceptor fluorescent dye" or simply as "acceptor" hereafter) such that fluorescence resonance energy transfer (also referred to as "FRET" hereafter) can take place to allow the detection of only the hybrid with the target nucleic acid; each of the probes is an oligonucleotide probe having a base sequence capable of hybridizing to the target nucleic acid adjacently with each other with the result of forming the hybrid.

[0007] Specifically, this invention is characterized in that it employs the detection probe having such a specified structure, and the following detection method is provided in accordance with the invention:

[0008] A method for detecting a target nucleic acid existing in cytoplasm of a living cell, the method comprising:

introducing into the cytoplasm, a detection probe bound to a nuclear membrane unpermeable molecule via a linker and labeled with a fluorescent dye, the probe having a base sequence capable of hybridizing to the target nucleic acid;
forming a hybrid between the target nucleic acid and the probe; and
determining any change in fluorescence of the fluorescent dye due to formation of the hybrid.

[0009] According to this invention, there is further provided:

[0010] The method for detecting a target nucleic acid existing in the cytoplasm of a living cell as described above, wherein the detection probe comprises a first probe member and a second probe member, the first and second probe

members have base sequences capable of hybridizing to the target nucleic acid adjacently with each other, the first probe member is labeled with an energy donor fluorescent dye and the second probe member is labeled with an energy acceptor fluorescent dye, and the change in fluorescence of the fluorescent dyes is fluorescence resonance energy transfer from the fluorescent dye of the first probe member to the fluorescent dye of the second probe member.

[0011] This invention provides the detection method as described above wherein the nuclear membrane unpermeable molecule is preferably at least one member selected from the group consisting of proteins, sugars, beads, and metal particles that have sizes sufficient so as not to pass through the nuclear membrane pores. More preferably, the protein includes streptavidin and avidine. Also, the sugar includes dextran.

[0012] In the detection method described above, the detection probe may further contain a molecule that blocks digestion of an oligonucleotid by nuclease (hereafter referred to as "nuclease-blocking molecule" and that is bound to the detection probe via a linker. Here, it is preferred that the nuclease-blocking molecule be identical with the nuclear membrane unpermeable molecule. This means that the nuclear membrane unpermeable molecule for use in the invention is provided with the function of a nuclease-blocking molecule.

[0013] This invention provides the detection method as described above wherein the nuclear membrane unpermeable molecule is characterized in that it is preferably at least one member selected from the group consisting of proteins, sugars, beads, and metal particles. More preferably, the protein includes streptavidin and avidine. Also, the sugar includes dextran.

[0014] In the detection method described above, the detection probe is preferably an oligonucleotide comprising from 10 to 20 bases.

[0015] Further, in the detection method described above the target nucleic acid to be detected is preferably messenger RNA (mRNA).

[0016] Since the detection method of the invention is highly specific, it will become possible to detect only the target nucleic acid with high sensitivity despite that a large number of nucleic acids of other kinds are present in a living cell.

[0017] The present invention will be more fully understood from the detailed description given hereinbelow and the accompanying drawings, which are given by way of illustration only and are not to be considered as limiting the present invention.

[0018] Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will be apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Fig. 1 A shows a structure for the probe to be used in this invention. Fig. 1 B is a schematic representation of the detection method of the invention.

[0020] Figs. 2A-2D are a set of fluorescence micrographs showing fluorescence images of a cell 10 minutes after introduction of a pair of fluorescently labeled probes into a Cos7 cell by microinjection. Fig. 2A shows a DD image obtained when the Bodipy493/503-labeled oligo DNA probe (D2F) was introduced into the cell; Fig. 2B shows an AA image obtained when the Cy5-labeled oligo DNA probe (A2F) was introduced into the cell; Fig. 2C shows a DD image obtained when the Bodipy 493/503-labeled oligo DNA probe bound to streptavidin (D2FB/streptavidin) was introduced into the cell; and Fig. 2D shows an AA image obtained when the Cy5-labeled oligo DNA probe bound to streptavidin (A2FB/streptavidin) was introduced into the cell.

[0021] Fig. 3 is a graph showing change in the fluorescence spectrum when c-fos RNA was added to the solutions of two pairs of fluorescently labeled probes. In the figure, "a" represents the spectrum obtained when D2F and A2F were mixed at a molar ratio of 1:1; "b" represents the spectrum obtained when c-fos RNA synthesized by *in vitro* transcription reactions was added to the mixed solution of D2F/A2F and incubated for 15 minutes (D2F:A2F:c-fos RNA = 1:1:1 (molar ratio)); "c" represents the spectrum obtained when D2FB/streptavidin and A2FB/streptavidin were mixed at a molar ratio of 1:1; and "d" represents the spectrum obtained when c-fos RNA was added to the mixed solution of D2FB/streptavidin and A2FB/streptavidin and incubated for 15 minutes (D2FB/streptavidin:A2FB/streptavidin:c-fosRNA = 1:1:1 (molar ratio)).

[0022] Fig. 4 is a graph showing change in the fluorescence intensity ratio (DA/DD) in fluorescence microscope images (DD images and DA images) with formation of a 3-member hybrid of the probe pair and target RNA. In the figure, a 40mer RNA with a base sequence complementary to the probes was mixed at different ratios with a pair of the D2FB/streptavidin and A2FB/streptavidin solutions (molar ratio of D2FB/streptavidin and A2FB/streptavidin=1:1), and the mixtures were incubated at room temperature for 15 minutes. The DD images and DA images were taken from the incubated solutions under a fluorescence microscope, and the ratios of the DD values and DA values (DA/DD) were plotted against the ratios of RNA to probes (RNA/probes).

[0023] Figs. 5A-5D are a set of fluorescence micrographs showing change in the fluorescence images of a cell into

which a pair of fluorescently labeled probes had been introduced, after a target RNA was introduced by injection into the cell. A solution of D2FB/streptavidin and A2FB/streptavidin mixed at a molar ratio of 1:1 was introduced into Cos7 cells by microinjection. Fig. 5A shows a DD image and Fig. 5B shows a DA image. The 40mer target RNA was also injected after 10 minutes. Fig. 5C shows the later DD image and Fig. 5D shows the later DA image. The length of the bar in Fig. 5A represents 20 μm.

[0024] Figs. 6A-6D are a set of fluorescence micrographs showing change in the fluorescence images of a cell into which a pair of fluorescently labeled probes had been introduced, after a non-target DNA was introduced by injection into the cell. A solution of D2FB/streptavidin and A2FB/streptavidin mixed at a molar ratio of 1:1 was introduced into Cos7 cells by microinjection. Fig. 6A shows a DD image and Fig. 6B shows a DA image. The 40mer non-target DNA was also injected after 10 minutes. Fig. 6C shows the later DD image and Fig. 6D shows the later DA image. The length of the bar in Fig. 6A represents 20 μm.

[0025] Fig. 7 is a graph showing the changes in DA/DD values in the cytoplasm plotted against time for the experiment of Figs. 5A-5D and 6A-6D. In the figure, "a" represents the cell of Figs. 5A-5D, and "b" represents the cell of Figs. 6A-6D.

[0026] Figs. 8A-8D are a set of fluorescence micrographs showing the time-dependent change in the fluorescence images of a mRNA-expressing cell after introduction of a pair of fluorescently labeled probes by microinjection. Here, the streptavidin-bound probes with no linker (D2FB, A2FB) were introduced into transfected a Cos7 cell. Fig. 8A shows the state after 1 minute; Fig. 8B shows the state after 5 minutes; Fig. 8C shows the state after 10 minutes; and Fig. 8D shows the state after 20 minutes. The length of the bar in Fig. 8A represents 20 μm.

[0027] Figs. 9A-9D are a set of fluorescence micrographs showing fluorescence images of an mRNA-expressing cell after introduction of a pair of fluorescently labeled probes by microinjection. Here, the streptavidin-bound linker-introduced probes (D2FBL, A2FBL) were introduced into a transfected Cos7 cell. Fig. 9A shows a DD image; Fig. 9B shows a DA image; Fig. 9C shows an AA image; and Fig. 9D shows a phase contrast (Ph) image. The length of the bar in Fig. 9A represents 20 μm.

[0028] Figs. 10A and 10B are pseudocolor representations of the ratio image obtained by dividing the DA image by the DD image in the experiment of Figs. 9A-9D. Fig. 10 A shows the state after 5 minutes, and Fig. 10B shows the state after 20 minutes.

[0029] Figs. 11A-11D are a set of fluorescence micrographs showing the time-dependent change in the fluorescence images of an mRNA-expressing cell after introduction of a pair of fluorescently labeled probes by microinjection. Here, D2FBL and A2FBL were introduced into a transfected Cos7 cell. Fig. 11A shows the state after 1 minute; Fig. 11B shows the state after 5 minutes; Fig. 11C shows the state after 10 minutes; and Fig. 11D shows the state after 20 minutes. The length of the bar in Fig. 11A represents 20 μm.

[0030] Fig. 12 is a graph showing DA/DD values for the cytoplasm of the cells used in the experiments of Figs. 8A-8D, 11A-11D, 13A-13D and 14A-14D plotted against time. In the figure, "a" represents the cell of Fig. 11A-11D, "b" the cell of Fig. 13A-13D, "c" the cell of Fig. 14A-14D and "d" the cell of Fig. 8A-8D.

[0031] Figs. 13A-13D are a set of fluorescence micrographs showing the time-dependant change in fluorescence images of an mRNA-expressing cell after introduction of a pair of fluorescently labeled probes by microinjection. Here, D2FBL and A2FBL were introduced into a non-transfected Cos7 cell. Fig. 13A shows the state after 1 minute; Fig. 13B shows the state after 5 minutes; Fig. 13C shows the state after 10 minutes; Fig. 13D shows the state after 20 minutes. The length of the bar in Fig. 13A represents 20 μm.

[0032] Figs. 14A-14D are a set of fluorescence micrographs showing the time-dependant change in fluorescence images of an mRNA-expressing cell after introduction of a pair of fluorescently labeled probes by microinjection. Here, the streptavidin-bound linker-introduced probes (D2SFBL and A2SFBL: sense sequence probes) were introduced into a transfected Cos7 cell. Fig. 14A shows the state after 1 minute; Fig. 14B shows the state after 5 minutes; Fig. 14C shows the state after 10 minutes; and Fig. 14D shows the state after 20 minutes. The length of the bar in Fig. 14A represents 20 μm.

[0033] Figs. 15A-15D are a set of fluorescence micrographs showing fluorescence images of an mRNA-expressing cell after introduction of a pair of fluorescently labeled probes by microinjection. Here, D2SFBL and A2SFBL were introduced into a transfected Cos7 cell. Fig. 15A shows a DD image; Fig. 15B shows a DA image; Fig. 15C shows an AA image; and Fig. 15D shows a Ph image.

[0034] Figs. 16A and 16B are pseudocolor representations of the ratio image obtained by dividing the DA image by the DD image in the experiment of Fig. 15A-15D. Fig. 16A shows the state after 5 minutes, and Fig. 15B shows the state after 20 minutes.

[0035] Fig. 17 is a graph showing DA/DD values for the cytoplasm in the individual cells injected with probes. Here, a solution of D2FBL and A2FBL mixed at 1 x 10^5 molecules each was introduced into transfected Cos7 cells by microinjection at 3-5, 24, 48 and 72 hours after transfection. As a control, D2FBL and A2FBL were injected into non-transfected cells (control 1) and D2SFBL and A2SFBL were injected into transfected cells (control 2). In the figure, the symbol "♦,●,or ▲" each represents the DA/DD value for the individual cell, and the symbol "□" represents the average DA/DD value for the group of cells at a particular time.

[0036] Fig. 18 is a graph showing the average number of molecules of c-fos mRNA expressed per cell after transfection, as the result of deduction by dot blotting, plotted against time.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0037] This invention will be explained in detail by referring to preferred embodiments.

Outline of the Detection Method of the Invention

[0038] As an example of the detection method of the invention, an explanation will be made below to the preferable procedure for visualizing and detecting a specified kind of mRNA within a living cell.

[0039] As Fig. 1 schematically shows, two types of probe for detection are provided and are combined to a pair for use. Each probe comprises three components: (1) an oligonucleotide that hybridizes to target mRNA; (2) a molecule having such a size that does not permeate through the nuclear membrane in a cell; (3) a linker connecting the two. The base sequence of each oligonucleotide constituting a first or a second probe member is a base sequence capable of hybridizing adjacently with each other to the specified site of mRNA which is the object of detection.

[0040] mRNA is generally believed to have a complex structure of its own. For this reason, even if the oligonucleotide has a base sequence complementary to the specified site of mRNA, steric hindrance to hybridization may occur at the particular site. This necessitates the selection of the site of mRNA at which the oligonucleotide should hybridize to the target mRNA. Selection of the specified sites is feasible, for example, in the following manner. First, the secondary structure of mRNA is simulated. For example, this can be done by using a commercially available computer program for prediction of the secondary structures of RNAs such as DINsis. In the obtained secondary structure map, a site comprising 30-40 bases centered around the site at which RNA forms a single strand structure (loop) is selected. The site is divided into two segments (each 15-20 bases). Oligonucleotides having base sequences complementary to the respective segments are synthesized. These two segments make up a first oligonucleotide and a second oligonucleotide.

[0041] Further, the first and second oligonucleotides are respectively labeled with fluorescent dyes of different types. These two types of fluorescent dye are selected to be a combination such that it causes FRET when they are located at an adequate distance (8 nm or less) with respect to each other: one dye is denoted "donor" and the other denoted "acceptor." The labeling positions of the respective fluorescent dyes on the oligonucleotide are those which allow for the stereostructure such that the two types of fluorescent dye are located at a distance at which they generate FRET when the two types of probe and the target mRNA form a 3-member hybrid. In practice, if the two fluorescent dyes are located within a distance of 20 bases or less on the hybrid, this condition will be met. More specifically, a model experiment has demonstrated that two to four bases efficiently cause FRET. For a general review on FRET, see Lakowicz, J. R. "Principles of Fluorescence Spectroscopy" (1983), Plenum Press, New York.

[0042] The first and the second fluorescently labeled oligonucleotides that have been synthesized in the manner described above are mixed, and their fluorescent spectra are measured. Next, the target mRNA is added to this solution, and any change in fluorescent spectrum is observed. If these three forms a hybrid, FRET occurs between the two types of fluorescent dye. The result is that one obtains a fluorescent spectrum where the fluorescent intensity of the donor has decreased and the fluorescent intensity of the acceptor has increased.

[0043] Preferably, the above-described manipulations are conducted on each of several kinds of site that are selected on the secondary structure map of mRNA; the degrees of spectral change are compared, and the sites showing large degrees of spectral change are selected. The mRNA to be used in the measurement can be synthesized by *in vitro* transcription reactions using a plasmid DNA into which has been incorporated the corresponding cDNA.

[0044] To accurately evaluate the efficiency of hybridization of the respective sites to the target mRNA, the following is to be done. After each fluorescently labeled oligonucleotide and the target mRNA are mixed in aqueous solution, the oligonucleotide having hybridized to the mRNA and the oligonucleotide not having hybridized to the mRNA are separated by HPLC. The ratio of the oligonucleotide having undergone hybridization is determined from the area ratio of the fluorescent intensities of the respective peaks.

[0045] It is also possible to confirm and evaluate the hybridization of each oligonucleotide to the target mRNA in a living cell by *"in situ* transcription method." Namely, the fluorescently labeled oligonucleotide is incorporated into the living cell. This can be done, for example, by adding the fluorescently labeled oligonucleotide to a medium where the cells are grown and by incubating the medium for a predetermined period. Subsequently, the cells are fixed. Reverse transcription is then conducted on the fixed cells, where the position of the oligonucleotide serves as the initiation site of a primer. If the oligonucleotide is hybridized to the target mRNA, the transcription will take place, thus producing cDNA corresponding to the target mRNA. The synthesized cDNA is then to be detected.

[0046] Next, each oligonucleotide is bound via a linker to a molecule having such a size that does not permeate through the nuclear membrane. For example, streptavidin, one kind of protein, can be used as the molecule having

such a size that does not permeate through the nuclear membrane; and an oligonucleotide can be used as the linker. In this case, it is preferred that the fluorescently labeled oligonucleotide for hybridization with the target mRNA and the oligonucleotide equipped with the function of a linker be synthesized as a single oligonucleotide. Biotin is coupled to one end of the oligonucleotide. The oligonucleotide labeled with a fluorescent dye and biotin is mixed with streptavidin in aqueous solution to achieve binding of the two. Since streptavidin is tetravalent with respect to biotin in binding, a plurality of fluorescently labeled oligonucleotide molecules may bind to one molecule of streptavidin. To prevent this from occurring, the mixing ratio is set such that streptavidin is present in excess. In practice, the molar ratio of oligonucleotide to streptavidin is desirably 1:4. Donor and acceptor probes are both used in the form of being bound to streptavidin. Therefore, it must be avoided that both of the donor and acceptor oligonucleotides are bound to the same streptavidin, for FRET occurs when the both are bound. The binding reaction between the oligonucleotide and streptavidin is conducted under the conditions where streptavidin is present in excess relative to each of the donor and acceptor oligonucleotides. This ensures that all the oligonucleotides be bound to streptavidin: no free oligonucleotide is present when the donor oligonucleotides premixed with streptavidin are mixed with the acceptor oligonucleotides premixed with streptavidin.

[0047] The length of the linker may be such that it effectively causes the relaxation of steric hindrance so that hybridization of the probes described above to the target mRNA in the cell progresses efficiently. Practically, a length of 20 bases or less is desirable. There are no particular limitations for the base sequence of the linker portion; however, one must avoid to use a base sequence that possibly causes hybridization of the target mRNA to other sites or hybridization to other kinds of mRNA. The backbone of the linker portion may employ one that is highly resistant to nuclease, such as S-oligo (deoxysulphonate oligonucleotide).

[0048] The two types of probe thus prepared are mixed and introduced into cells. For example, the probes may be injected into the cytoplasm by microinjection.

[0049] The fluorescent image of the cell is to be measured. The fluorescent image of the cell is observed with a standard inverted fluorescence microscope. The excitation filter, dichroic filter and fluorescence filter are adequately set to the wavelength of the donor dye and to that of the acceptor dye, respectively. This permits observation of the distribution of the donor probe within the cell, the distribution of the acceptor cell within the cell, and their time-dependent changes. The cells are incubated on a stage of the microscope. Where necessary, among others the temperature is regulated, for example, by the method circulating the culture medium.

[0050] Measurement of FRET in a cell can be conducted by determining the ratio of fluorescent intensities in two wavelength regions. Specifically, where the efficiency of FRET at an arbitrary position in the cell is to be determined, the fluorescent intensity (Id) in the wavelength region of donor fluorescence and the fluorescent intensity (Ia) in the wavelength region of acceptor fluorescence are measured at that position when the cell is irradiated with light in the excitation wavelength region of the donor dye; and the ratio (Ia/Id) of the Ia value to the Id value may be determined. The value of Ia/Id represents the efficiency of FRET.

[0051] Fluorescent images in two wavelength regions, namely a fluorescent image taken in the wavelength region of the donor fluorescence (referred to as "DD image" throughout the specification) and a fluorescent image taken in the wavelength region of the acceptor fluorescence (referred to as "DA image" throughout the specification) by irradiating the cell with the light in the excitation wavelength region of the donor, are obtained. Imaging of FRET in the cell becomes possible by dividing the DA image by the DD image to obtain an image (referred to as "DA/DD image" throughout the specification unless the term "the ratio image" is used instead).

[0052] Measurement of FRET can also be possible through the time-resolved measurement of fluorescence. That is, the cell is irradiated with the light in the excitation wavelength region of a donor dye in a pulse manner, and the decay curve of fluorescence intensity in the wavelength region of the donor fluorescence or in the wavelength region of the acceptor fluorescence is measured. When FRET occurs, it accelerates the rate of the fluorescence decay of the donor and delays the fluorescence decay of the acceptor. Measurement of the rate of fluorescence decay under microscopy can be made, for example, by using a camera with a time-gate function, setting two time zones for measurement within the time period when the fluorescence decay is occurring, and by determining fluorescence intensities in the respective time zones to obtain the ratio of the fluorescence intensities. To effectively detect the formation of hybrids through the time-resolved measurement, fluorescently labeled oligonucleotides may be used that satisfy the conditions disclosed in PCT/JP97/03438.

[0053] The detection method of this invention will be explained in detail hereafter.

Target Nucleic Acids

[0054] There are no particular limitations for the kind of nucleic acid in cytoplasm that can be detected by the detection method of the invention. Any kind of nucleic acid that can be detected by hybridization methods in the prior art or a derivative thereof may be included. Concretely, there can be mentioned DNA and RNA. Particularly, the RNA includes mRNA.

[0055] There are also no particular limitations for the structure possessed by a target nucleic acid that can be detected by the detection method of the invention; and it may be one that has a structure as its portion to which hybridization methods in the prior art are applicable. Namely, the target nucleic acid is the one at least a part of the base sequence of which is known. When the base sequence of a target nucleic acid is unknown, a part or its entire base sequence can be readily determined according to a variety of base sequencing well known in the art. The detection probes that will be discussed below hybridize to such base sequences in a complementary manner. There are also no particular limitations for the number of bases in the base sequence, and it can readily be selected by referring to the conditions known in hybridization methods in the prior art.

[0056] The target nucleic acids that can be detected by the detection method of the invention are not limited to those already existing in cytoplasm when the detection probe is introduced into the cytoplasm. The nucleic acids can be detected even if they are produced and come to being within the cytoplasm after the detection probe has been introduced into the cytoplasm.

Detection Probes

[0057] The detection probe that can be used in the detection method of the invention has at least the following features: (1) an oligonucleotide structure that has a base sequence capable of hybridizing to at least a part of the base sequence of the target nucleic acid, as is similarly required by the hybridization methods in the prior art; (2) a labeling molecule that only allows the detection of the hybrid formed by hybridization with the target nucleic acid; (3) a nuclear membrane unpermeable molecule that prevents the permeation of an oligonucleotide through nuclear membrane pores; and preferably (4) a nuclease-blocking molecule that prevents an oligonucleotide from being digested by nuclease. Thus, any oligonucleotide probe is usable without any particular limitations provided that it has the above-mentioned features. These features will be explained in more detail hereafter.

1. Oligonucleotide Structure

[0058]

A. There are no particular limitations for the kind or the base number of base sequence capable of hybridizing to at least a part of the base sequence of the target nucleic acid as explained above. Preferably, applicable are the conditions that may be used in standard hybridization methods. Based on the specified sequence of a target nucleic acid, base sequences complementary thereto may readily be selected. Further, it is also possible to adequately select the complementation depending on the hybridization conditions.

For the detection probe of this invention, optimum hybridization conditions may be set by referring to the temperature of a sample or the concentration of a target nucleic acid existing in the sample; on that basis the base number may be selected. For example, the melting point of a hybrid formed among the oligonucleotide probes and the target nucleic acid increases with increasing numbers of bases of the probes. The hybrid is formed with sufficient high efficiency at room temperature when the base number of the probe is 15, while the efficiency of formation of the hybrid at 37 °C is low. To perform detection at 37 °C, it is therefore desirable to use an oligonucleotide having a length of 20 bases or more as the probe. On the other hand, as the base number of the oligonucleotide probe grows greater, the reaction rate by which the probe forms the hybrid with the target nucleic acid becomes lower. Concretely, the time required for the hybridization reaction between DNA probes with 20 bases and the target nucleic acid to be complete is a few times that needed when DNA probes with 15 bases are used. Judging from these factors, the base number of the oligonucleotide probe for use is preferably in the range of from 10 to 50 bases, more preferably in the range of from 15 to 20 bases. If the base number falls short of such range, there will be difficulty of the formation of a sufficiently stable hybrid; on the other hand, if the number exceeds the range, there may be cases where problems arise from various aspects such as preparation of the detection probes, their stability, and the times required for the hybrid formation. Concerning the complementation of the base sequences between the detection probes and the target nucleic acid, the most preferred is a sequence with complete complementation. In the case of an oligonucleotide, probe for detection having no complementation in at least a portion thereof, the melting point of the hybrid with the target nucleic acid experiences a decrease as compared to the one with complete complementation.

B. There are no particular limitations for the other structure of the detection probe having an oligonucleotide structure with the base sequence as explained in A above.

C. Where necessary, the detection probe can possibly be used such that hybrids are formed at a plurality of sites in the target nucleic acid. For example, the sites may be a position near one end of the target nucleic acid, a position near the middle point thereof, and a position near the other end thereof.

2. Labeling Molecules

[0059] The detection method of this invention detects a target nucleic acid in cytoplasm by detecting the hybrid of the target nucleic acid and detection probes. Particularly, in the invention it will be possible to detect the target nucleic acid existing in the cytoplasm of a living cell; for this reason, it is preferred that the invention can detect only the hybrid being formed without the need of its separation. Therefore, the labeling molecules preferably utilize the phenomenon that results only when they are hybridized to the target nucleic acid adjacently with each other. There are no particular limitations for such a phenomenon, and a variety of phenomena that are well known in the prior art may be relied upon. Especially, in this invention it is preferable to utilize fluorescence phenomena in view of their high detection sensitivity, means for their easy measurement, and the like. Specifically, the phenomenon that only results from the molecules' being hybridized is preferably one that utilizes the phenomenon, "FRET." Such labeling by fluorescence will be made possible by labeling a pair of single strand oligonucleotide probes with an energy donor fluorescent dye and an energy acceptor fluorescent dye. In this regard, there are no particular limitations for the donor and acceptor molecules that can cause FRET; and one skilled in the art finds it easy to adequately select among the already known combinations of fluorescent dyes. Concretely mentioned as a donor are fluorescent dyes of the Bodipy type (4,4-difluoro-4-bora-3a, 4a-diaza-S-indacene, Molecular Probe), of the fluorescein type, and of the Rhodamine type. Mentioned as an acceptor are fluorescent dyes of the indocyanine type, and of the Rhodamine type. For the combination of donor and acceptor molecules that can preferably be used in this invention, there may be mentioned a Bodipy/indocyanine type. Preferable individual fluorescent dyes are Bodipy493/503 as the donor and Cy5 as the acceptor. This is because the fluorescence spectrum of the former is almost separated from the fluorescence spectrum of the latter at wavelength.

3. Nuclear Membrane Unpermeable Molecules

[0060] The detection probe explained in 2 above has a molecule that prevents the probe in the cytoplasm from permeating into the nucleus through nuclear membrane pores. Particularly, as the molecule that does not pass through nuclear membrane pores by diffusion, a molecule can be selected such that it is large sufficient for the sizes of the nuclear membrane pores. Furthermore, there are no particular limitations for the components that comprises the nuclear membrane unpermeable molecule, and a variety of biological and synthetic components can be used. The biological components or the components based thereon include proteins and dextran. The synthetic components include beads made of various constituents and metal particles such as colloidal gold.

[0061] There are no particular limitations to the method for binding a nuclear membrane unpermeable molecule to the oligonucleotide explained in 1. above, and binding methods ordinarily known in the art are applicable. Specifically, when a protein is selected as the nuclear membrane unpermeable molecule, binding is preferably done through reaction with amino acid residues (e.g., amino or carboxyl) within the protein. Specific binding between biotin and streptavidin or avidine may also be utilized. Namely, biotin is incorporated into one end of an oligonucleotide or within a strand thereof in synthesis, avidine or streptavidin is mixed with the product. Through this manipulation the oligonucleotide containing biotin is allowed to bind to avidine or streptavidin. The oligonucleotide containing biotin can readily be synthesized on a DNA synthesizer. Further, the nuclear membrane unpermeable molecule is bound to the oligonucleotide via a linker of appropriate length. Such linkers include oligonucleotide derivatives and oligopeptides.

4. Nuclease-Blocking Molecules

[0062] Preferably, the detection probe explained in 2 above has a nuclease-blocking molecule that prevents an oligonucleotide from being digested by nuclease. Although there are no particular limitations for the blocking, this invention preferably employs such molecules that prevent a substrate from approaching to its binding site on an enzyme (nuclease) by virtue of steric hindrance. Furthermore, there are no particular limitations for the components that comprise the nuclease-blocking molecule, and a variety of biological and synthetic components can be used. The biological components or the components based thereon include proteins and dextran. The synthetic components include beads made of various constituents and metal particles such as colloidal gold.

[0063] There are no particular limitations to the method for binding a nuclease-blocking molecule to the oligonucleotide explained in 1 above, and binding methods ordinarily known in the art are applicable. Specifically, when a protein is selected as the nuclease-blocking molecule, binding is preferably done through reaction with amino acid residues (e.g., amino or carboxyl) within the protein. Specific binding between biotin and streptavidin or avidine may also be utilized. Namely, biotin is incorporated into one end of an oligonucleotide or within a strand thereof in synthesis, avidine or streptavidin is mixed with the product. Through this manipulation the oligonucleotide containing biotin is allowed to bind to avidine or streptavidin. The oligonucleotide containing biotin can readily be synthesized on a DNA synthesizer. Further, where necessary, the nuclease-blocking molecule is bound to the oligonucleotide via a linker of appropriate length. Mentioned as such a linker are oligonucleotide derivatives and oligopeptides. As used herein, the oligonucle-

otide includes D-oligo (deoxyoligonucleotide) and S-oligo.

[0064] Particularly in this invention, it is preferred that the nuclear membrane unpermeable molecule explained above be a nuclease-blocking molecule at the same time. That is, the nuclear membrane unpermeable molecule prevents the detection probes from permeating through nuclear membrane pores, while at the same time, it prevents the detection probes or the hybrids of the detection probes and the target nucleic acid from enzymatic digestion. In such cases, it is preferred that the nuclear membrane unpermeable molecule effectively exerting the two kinds of inhibition (synergic nuclease-blocking molecule) be provided with a linker of appropriate type and length. For the type of linker, concretely mentioned are oligonucleotides and oligopeptides, the former of which are particularly preferred. If the linker is too long, it will not produce steric hindrance that prevents the enzymatic reaction, for which reason its length is preferably shorter than about 20 bases. On the other hand, if the linker is too short, its steric hindrance will obstruct the formation of a stable hybrid with the target nucleic acid. Consequently, the kind and length of a suitable linker can be adequately selected based on steric molecular models, molecular model calculation programs, and the like.

Detection Methods

[0065] According to a preferred embodiment of this invention, the detection probes explained above are introduced into the cytoplasm and allowed to hybridize to the target nucleic acid, forming the hybrid; at that time, the probes are irradiated with the excitation light of the donor fluorescent dye bound to the probe to cause FRET, and fluorescence from the acceptor fluorescent dye due to the FRET is observed. That is, the donor fluorescent dyes of the detection probes that have not formed hybrids are simultaneously irradiated, but FRET does not occur between the acceptor fluorescent dyes of the detection probes that have not formed hybrids and the donor fluorescent dyes. Thus, fluorescence from the acceptor fluorescent dyes can not be observed. Consequently, the fluorescence from acceptor fluorescent dye means the presence of a hybrid, namely the presence of the target nucleic acid.

[0066] Moreover, there are no particular limitations for the detection device to be used in the detection method of this invention, and it may be sufficient provided that it can excite the donor fluorescent dye and can measure fluorescence of the acceptor fluorescent dye as well. Specifically the use of a fluorescence microscope is mentioned. There are also no particular limitations for the method of measurement, and mentioned are the method relying on the measurement of fluorescence intensity and the method relying the time-resolved measurement.

[0067] There are also no particular limitations for the method of introducing the detection probes into the cytoplasm, and introduction methods well known in the prior art may be used. Concretely mentioned are microinjection and the introduction method which relies on as a carrier, a transfection reagent such as lipofectoamine.

[0068] In what follows, the detection method of this invention will be explained in more detail based on the example where mRNA of human c-fos gene was actually detected in Cos7 cells; however, the invention is not to be limited to the particular example. Here, a combination of Bodipy493/503 as the donor and Cy5 as the acceptor was used.

EXAMPLES

(Production Example 1) Preparation of Fluorescently Labeled Oligodeoxynucleotide Probes

1. Prediction of Secondary Structure of c-fos mRNA

[0069] A base sequence of human c-fos cDNA was searched in the DNASIS database (Hitachi Software Engineering). PolyA tail was added to the base sequence to prepare a c-fos mRNA sequence. The DNASIS software (Hitachi Software Engineering) was used on this sequence for simulation of the secondary structure of c-fos mRNA. From the resulting secondary structure map, there were selected four domains that showed a loop structure (single stranded structure), and a 40mer site containing the loop structure was selected for each domain. These sites were base Nos. 206-245, 657-696, 898-937 and 1659-1698.

2. Synthesis of Oligodeoxynucleotides

[0070] Each of the four selected sites was divided into two 20mer halves, and oligodeoxynucleotides (referred to as "oligo DNA(s)" throughout the specification) complementary to the respective sequences were synthesized.

D1: Oligo DNA with base sequence complementary to 206-225

5'-XGAACATCATCGTGGCGGTTA-3'

A1: Oligo DNA with base sequence complementary to 226-245

5'-TAGTCTGCGTTGAAGCYCCGA-3'

D2: Oligo DNA with base sequence complementary to 657-676

5'-XTCTAGTTGGTCTGTCTCCGC-3'

A2: Oligo DNA with base sequence complementary to 677-696

5'-GCAAAGCAGACTTCTCYATCT-3'

D3: Oligo DNA with base sequence complementary to 898-917

5'-XTCCGGGGTGGCAACCTCTGG-3'

A3: Oligo DNA with base sequence complementary to 918-937

5'-GGGTGAAGGCCTCCTCYAGAC-3'

D4: Oligo DNA with base sequence complementary to 1659-1678

5'-XAAGGACTAAGGAGAAAGAGA-3'

A4: Oligo DNA with base sequence complementary to 1679-1698

5'-AGATTAGTTAATGCTAYTGAG-3'

[0071] The synthesis of each of these oligo DNAs was carried out according to the β-cyanoethyl amidite method, using a Model 394 (Perkin Elmer Applied Biosystems) or an Expeptide Model 8909 (Perspective). Here, "X" was 6-(trifluoroacetylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoroamidite (TFAc hexanolamine linker, Perkin Elmer, Japan: Cat. No. 400808), and "Y" was Uni-Link Amino Modifier (CLONTECH, Code No. CL5190-1).

[0072] The resulting crude product was analyzed by DEAE-HPLC and the main peak fractions were recovered. The retention times were 20-30 minutes. The aliquots were desalted and then lyophilized.

DEAE-HPLC (anion-exchange) conditions
Solvent A: 0.2 M $HCOONH_4$, 20% CH_3CN
Solvent B: 1.0 M $HCOONH_4$, 20% CH_3CN
Column: TSK-gel DEAE-2WS; 4.6 x 250 mm (Tosoh)
Flow rate: 0.8 ml/min
Temperature: 40 °C
B gradient: 35-85% (20 min)

3. Fluorescence Labeling of Oligodeoxynucleotides

3-1. Labeling with Bodipy493/503

[0073] The fluorescent dye Bodipy493/503 was conjugated to the "X" of each oligo DNA of D1, D2, D3 and D4 by the following method.

[0074] Separately there were dissolved 2.5 mg of sodium N-hydroxysulfosuccinimide in 30 µl of sterilized water and 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in 50 µl of sterilized water. Bodipy493/503 propionic acid, 1 mg, (Molecular Probes) dissolved in 50 µl of DMF was mixed therewith for reaction at room temperature for 30 min. The resulting solution was mixed with a solution of the oligo DNA, which was prepared by dissolving dried oligo DNA in 300 µl of 0.5 M $NaHCO_3/Na_2HCO_3$ (pH 9.0), and the mixture was allowed to react overnight with light exclusion. The reaction solution was applied to gel filtration and the unreacted dye was eliminated. Analysis was performed by reversed phase HPLC, and the peak at 25-35 minutes was recovered.

Reversed phase HPLC conditions
Solvent A: 0.05 M TEAA (triethylammonium acetate), 5% CH_3CN
Solvent B: 0.05 M TEAA, 40% CH_3CN
Column: CAPCELL PAK C18; 6 x 250 mm (Shiseido)
Flow rate: 1.0 ml/min
Temperature: 40 °C
B gradient: 30-80% (20 min)

[0075] The samples were lyophilized and stored. Just prior to use, they were dissolved in DEPC-water (diethyl pyrocarbonate) and then diluted with 1 x SSC solution; and their absorption spectra were measured. The Bodipy493/503 labeling yields (Bodipy 493/503/oligo DNA) were determined by the ratios of the 260 nm absorbance and the 504 nm absorbance. The Bodipy493/503/oligo DNA values were 0.8-0.9 (molar ratio).

[0076] The resulting Bodipy493/503-labeled oligo DNAs were as follows.

D1F: 5'-(Bodipy493/503)GAACATCATCGTGGCGGTTA-3'

D2F: 5'-(Bodipy493/503)TCTAGTTGGTCTGTCTCCGC-3'

D3F: 5'-(Bodipy493/503)TCCGGGGTGGCAACCTCTGG-3'

D4F: 5'-(Bodipy493/503)AAGGACTAAGGAGAAAGAGA-3'

3-2. Labeling with Cy5

[0077] .Cy5 was conjugated to "Y" of each oligo DNA of A1, A2, A3 and A4 by the following method.

[0078] FluoroLink Cy5 Mono Reactive Dye (Amersham Pharmacia Biotech) was dissolved in 100 µl of sterilized water, and this was then mixed with an oligo DNA dissolved in 200 µl of a 0.5 M $NaHCO_3/Na_2HCO_3$ buffer (pH 9.0) and was allowed to react overnight with light exclusion. The reaction solution was applied to gel filtration and the unreacted Cy5 was eliminated, after which analysis was performed by reversed phase HPLC, and the peak at 20-25 minutes was recovered.

Reversed phase HPLC conditions:
Solvent A: 0.05 M TEAA, 5% CH_3CN
Solvent B: 0.05 M TEAA, 40% CH_3CN
Column: CAPCELL PAK C18; 6 x 250 mm (Shiseido)
Flow rate: 1.0 ml/min
Temperature: 40 °C
B gradient: 15-65% (20 min)

[0079] The samples were lyophilized and stored. Just prior to use, they were dissolved in DEPC-water and then diluted with 1 x SSC buffer; and their absorption spectra were measured. The Cy5 labeling yields (Cy5/oligo DNA) were determined by the ratios of the 260 nm absorbance and the 649 nm absorbance. The Cy5/oligo DNA values were 0.9-1.0 (molar ratio).

[0080] The resulting Cy5-labeled oligo DNAs were as follows.

A1F: 5'-TAGTCTGCGTTGAAGC(Cy5)CCGA-3'

A2F: 5'-GCAAAGCAGACTTCTC(Cy5)ATCT-3'

A3F: 5'-GGGTGAAGGCCTCCTC(Cy5)AGAC-3'

A4F: 5'-AGATTAGTTAATGCTA(Cy5)TGAG-3'

(Preparation Example 2) Synthesis of c-fos RNA

[0081] c-fos RNA was synthesized by *in vitro* transcription reactions. Human c-fos DNA was obtained from the Riken Gene Bank in the form of a pSPT plasmid containing the full-length of c-fos cDNA (pSPT-cFos). The pSPT-cFos was treated with restriction enzyme EcoRI to cut out a c-fos DNA (2.1 kb). The c-fos DNA fragment was then incorporated into the EcoRI site of pBluescript KII(+) plasmid (Stratagene). The obtained plasmid (pBluescript-cFos) was linearized by treatment with restriction enzyme SmaI, and then used as a template for *in vitro* transcription reactions. The *in vitro* transcription reactions driven by T3 RNA polymerase were performed using a T3 MEGAscript kit (Ambion). The reaction solution was treaed with phenol/chloroform to extract the synthesized RNA from the solution containing proteins, followed by treatment with ethanol to precipitate and recover the synthesized RNA.

(Example 1) Assay for Hybridization of Fluorescently Labeled Probes to c-fos RNA

1. Fluorescently labeled oligo DNA probes (fluorescently labeled probes)

[0082] Each pair of oligonucleotides, D1F/A1F, D2F/A2F, D3F/A3F and D4F/A4F was used as a pair of fluorescently labeled probe for each site that showed a loop structure (single stranded structure) in the secondary structure prediction map for c-fos mRNA. These had base sequences complementary to the 206-225 site (D1F) and 226-245 site (A1F), the 657-676 site (D2F) and 677-696 site (A2F), the 898-917 site (D3F) and 918-937 site (A3F) and the 1659-1678 site (D4F) and 1679-1698 site (A4F) of c-fos mRNA, respectively. Upon forming a 3-member hybrid of each donor probe (Bodipy493/503-labeled oligo DNA) and acceptor probe (Cy5-labeled oligo DNA) with the c-fos RNA, the hybrid takes a form in which four nucleotides exist between the nucleotide to which are bound Bodipy493/503 and Cy5 linked, respectively.

2. Measurement of Fluorescence Spectra

[0083] D1F and A1F were mixed in 1 x SSC buffer each at a 1 x 10^{-6} M concentration, and the fluorescence spectrum was measured. The fluorescence spectrum was measured in the range of from 500 to 750 nm using an F4500 fluorescence spectrophotometer (Hitachi), with excitation at 480 nm.

[0084] The resulting spectrum has a peak near 514 nm. This corresponds to the fluorescence peak of Bodipy493/503. Little fluorescence was detected at 650-700 nm, where the fluorescence of Cy5 appears. c-fos RNA was then added to give a concentration of 1 x 10^{-6} M (D1F:A1F:c-fos RNA = 1:1:1 (molar ratio)). After reaction at room temperature for 15 min, the fluorescence spectrum was measured. The same experiment was carried out for D2F/A2F, D3F/A3F and D4F/A4F, respectively. When the pair of D2F/A2F was used as the fluorescently labeled probes, addition of the c-fos RNA resulted in a significant change in the spectrum. That is, the peak at 514 nm (fluorescence of Bodipy493/503) decreased and fluorescence with a peak at 668 nm (fluorescence of Cy5) appeared. The ratio of the fluorescence intensity at 668 nm and the fluorescence intensity at 514 nm was 0.015 with the probe alone, and 0.55 with the addition of c-fos RNA. A similar change was observed when D1F/A1F was used, but the change was smaller. When D3F/A3F and D4F/A4F were used, little change occurred in the spectrum by adding of c-fos RNA. These results are summarized in the following table.

Table 1

Ratio of Fluorescence Intensity at 514 nm (I(514)) and Fluorescence Intensity at 668 nm (I(668))		
[I(668)/I(514)j in the Fluorescence Spectra:		
fluorescently labeled probe types	probes alone (before addition of c-fos RNA)	after addition of c-fos RNA
D1F/A1F	0.015	0.15
D2F/A2F	0.015	0.55
D3F/A3F	0.015	0.08
D4F/A4F	0.015	0.09

[0085] The decrease in Bodipy493/503 fluorescence intensity and increase in Cy5 fluorescence intensity after addition of the c-fos RNA indicates hybridization of the two types of fluorescently labeled probes to the c-fos RNA occurred, resulting in FRET from Bodipy493/503 to Cy5. Based on these results, the 657-696 site (probe: D2F/A2F) was selected as the region for hybridization of oligonucleotides to c-fos RNA.

3. Confirmation of Hybridization between c-fos RNA and Fluorescently Labeled Probes by HPLC

[0086] In order to confirm hybridization of D2F and A2F to c-fos RNA, each of the fluorescently labeled probes was mixed with c-fos RNA in 1 x SSC solution and then used for an experiment whereby the c-fos RNA-hybridized probes and non-hybridized free probes were separated by high-performance liquid chromatography (HPLC). D2F or A2F and c-fos RNA were mixed in 1 x SSC (pH 7.0) solution at a molar ratio of 1:1 (2 μM each) and allowed to stand at room temperature for 20 min, and then analyzed by high-performance liquid chromatography (HPLC) using an ion-exchange column.

Column: DEAE-NPR (Tosoh)

Temperature: 25 °C
Flow rate: 1 ml/min
Mobile phase: 10 mM Tris-HCl, pH 9.5, 1 mM EDTA
Gradient: NaCl, 0.3-1 M (10 min)
Detection: ultraviolet absorption (260 nm)
Fluorescence detection: 475 nm excitation/515 nm fluorescence
650 nm excitation/667 nm fluorescence

[0087] The free probes were eluted at 3.5-4 min, and the c-fos RNA eluted at 6.9-7.1 minutes. Upon measuring the elution pattern for D2F by Bodipy493/503 fluorescence, peaks were observed at the free oligo DNA position and the c-fos RNA position. Based on the area integration values for the peaks, the ratio of the c-fos RNA to the coeluted D2F probe was 76%. When the Cy5 fluorescence was determined in the same manner for the A2F elution pattern, 43% of A2F was eluted at the c-fos RNA position. These results indicate that 76% of D2F and 43% of A2F hybridized to c-fos RNA when each probe was mixed with c-fos RNA at room temperature at a molar ratio of 1:1.

4. Effect of Probe Length on Hybridization

[0088] The effect of the fluorescently labeled probe length on hybridization at 37 °C was investigated. A pair of the 20mer D2F and A2F and a pair of a 15mer donor probe (Bodipy493/503-labeled oligo DNA) having the base sequence complementary to the 662-676 site (D5F) and a 15mer acceptor probe (Cy5-labeled oligo DNA) having the base sequence complementary to the 677-691 site (A5F) of c-fos mRNA were used.

[0089] The fluorescently labeled oligo DNAs used were the following. The synthesis and purification were carried out by the method described above.

D5F: 5'-(Bodipy493/503)TCTAGTTGGTCTGTC-3'

A5F: 5'-GCAGACTTCTC(Cy5)ATCT-3'

[0090] The D2F/A2F pair were mixed in 1 x SSC buffer at 1 x 10^{-6} M concentration each, and the fluorescence spectrum was measured. c-fos RNA was then added thereto to give a concentration of 1 x 10^{-6} M (D2F:A2F:c-fos RNA = 1:1:1 (molar ratio)). The same experiment was carried out for the D5F/A5F pair. These hybridization tests were repeated at 37°C. In all cases, hybridization was completed within 5-15 min. The change in the fluorescence spectrum after 15 min at room temperature was larger when D2F/A2F were used than when D5F/A5F were used. Results are summarized in Table 2.

[0091] The ratio of the fluorescence intensity at 668 nm (I(668)) and the fluorescence intensity at 514 nm (I(514)) represents the ratio of the fluorescently labeled probes that hybridizes to the target nucleic acid (c-fos RNA). The ratio of hybridizing probes was larger when D2F/A2F were used than when D5F/A5F were used. When the temperature was raised from room temperature to 37 °C, the ratio of hybridizing probes decreased in both cases. However, the decrease was relatively small for D2F/A2F, while it was considerable for D5F/A5F. Thus, the 20mer was shown to be preferable for use as hybridization probes in living mammalian cells.

Table 2

probe length	temperature	I(668 nm)/I(514 nm)
15mer/20mer	room temperature/37 °C	0.15 (in the absence of the target c-fos RNA
15mer	room temperature	0.40
15mer	37 °C	0.22
20mer	room temperature	0.51
20mer	37 °C	0.42

(Example 2) Assay for Hybridization of Fluorescently Labeled Probes to c-fos mRNA in Cells

1. Introduction of Fluorescently Labeled Probes into Cells by Injection and Observation of Cells under a Fluorescence Microscope

[0092] The D2F probe and A2F probe were mixed in sterilized water at 1 x 10^{-5} M concentration each, and a mixed solution of fluorescently labeled probes of the two types was introduced into Cos7 cells by microinjection.

[0093] Cos7 cells that had been plated for half a day in a dish with a cover glass on the bottom (P35G-O-14-C, MatTek Corp.) were used in the experiment. Just prior to the experiment, the culture solution was removed, and the cells were washed 3 times with HBSS solution and then observed with an inverted fluorescence microscope. The temperature of the cells on the microscope stage was controlled by circulating HBSS solution at 37 °C.
Microscope: Carl Zeiss Axiovert 135TV, objective lens: 40x Plan-Neofluar (phase contrast, NA = 0.75, Model No. 4403519902)

[0094] Microinjection was performed using an Eppendorf Micromanipulator 5171 or an Eppendorf Transjector 5246 Plus/BASIC, with a femtotip (Eppendorf). To minimize photobleaching during the measurement, the excitation light was attenuated with a 3% ND filter.

Excitation light source: Superhigh pressure mercury lamp (Model No. L4002)
Filters:
Bodipy493/503 fluorescence image (DD image)

Excitation filter: BP450-490, dichroic mirror: FT510
Barrier filter: BP515-565

Cy5 fluorescence image (AA image)

Excitation filter: BP575-625, dichroic mirror: FT645
Barrier filter: BP660-710

[0095] The fluorescence images were taken with a cooled CCD camera (Hamamatsu Photonics: C4880) and transferred into an image processing and analysis apparatus (Hamamatsu Photonics: ARGUS-50).

[0096] Most of both fluorescently labeled probes (D2F, A2F), which were introduced into the cytoplasm by injection, moved to and accumulated in the nucleus within 10 min (Fig. 2(a),(b)).

2. Distribution of Streptavidin-bound Oligo DNAs in Cells

[0097] Oligo DNAs each doubly labeled with a fluorescent dye and biotin were synthesized by the following procedure.

5'-XTCTAGTTGGTCTGTCTCCGCV-3'

5'-WGCAAAGCAGACTTCTCYATCT-3'

[0098] "X" is TFAc hexanolamine linker (Perkin Elmer, Japan), "Y" is Uni-Link Amino Modifier (CLONTECH), "V" is Biotin ON CPG (CLONTECH), and "W" is Biotin amidite (Perkin Elmer Biosystems). The synthesis and purification were carried out by the method described above. The above-mentioned method was used to bind Bodipy493/503 to "X" and Cy5 to "Y" to obtain the following oligo DNAs doubly labeled with the fluorescent dyes and biotin.

D2FB: 5'-(Bodipy493/503)TCTAGTTGGTCTGTCTCCGC(Biotin)-3'

A2FB: 5'-(Biotin)GCAAAGCAGACTTCTC(Cy5)ATCT-3'

[0099] These fluorescent/biotin-labeled oligo DNAs were bound to streptavidin. Streptavidin, 100 μg, (Molecular Probes, Cat. No. S-2669) was dissolved in 10 μl of PBS. To this was added D2FB (10^{-4} M) or A2FB (10^{-4} M) dissolved in DEPC-water to give a streptavidin/oligo DNA molar ratio of 4:1, and the mixtures were allowed to stand at room temperature for 10 min. The D2FB/streptavidin solution was then mixed with the A2FB/streptavidin solution.

[0100] The streptavidin-bound fluorescently labeled oligo DNA solution was injected into Cos7 cells. Observation of the Bodipy493/503 fluorescence image and Cy5 fluorescence image of the cells revealed localization of both the D2FB/streptavidin probe and the A2FB/streptavidin probe in the cytoplasm (Fig. 2C, 2D). No change in distribution of the probes occurred after incubating the injected cells for one hour on the microscope stage. These results demonstrated that the streptavidin-bound oligo DNA probes localize in the cytoplasm and do not move to the nucleus. Furthermore, if the oligo DNA is digested by nucleases present in the cell, less than 20mer fluorescently labeled oligo DNA fragment is released from the streptavidin. The released oligo DNAs would rapidly move to and accumulate in the nucleus. Thus, the fact that the fluorescence intensity of the nucleus remains almost unchanged during one hour after injection indicates that little digestion of the streptavidin-bound oligo DNAs has occurred.

[0101] The effect of streptavidin on hybridization of the fluorescently labeled probes to c-fos RNA was then investigated. Each 1 x 10^{-6} M of streptavidin-bound fluorescently labeled probes (D2FB/streptavidin, A2FB/streptavidin) was mixed with 1 x 10^{-6} M of c-fos RNA synthesized by *in vitro* transcription reactions in 1 x SSC solution (molar ratio of 1:1) and the mixture was allowed to stand at room temperature for 15 min; and then the fluorescent spectra were measured. The same measurement was made using the fluorescently labeled probes (D2F, A2F) not bound to streptavidin, as a control experiment (Fig. 3). In all of the samples, addition of c-fos RNA decreased the Bodipy493/503 fluorescence intensity and increased the Cy5 fluorescence intensity. The degree of change was slightly lower with the streptavidin-bound fluorescently labeled probes. This suggests a small degree of steric hindrance by streptavidin on hybrid formation of the probes with c-fos RNA.

3. Preparation of c-fos mRNA-expressing Cells

[0102] pSPT-cFos was treated with restriction enzyme EcoRI to cut out c-fos DNA (2.1 kb), and this was inserted into the EcoRI site of a pME18S expression vector (pME18S-cFos). The resulting expression vector (pME18S-cFos) was introduced into Cos7 cells by electroporation. The Cos7 cells were cultured in DMEM medium containing 10% fetal bovine serum, under conditions of 5% CO_2, 37 °C. The Cos7 cells were removed off with a cell cleaver and washed with PBS, and then 5 μg of pME18S-cFos was added to the cell suspension (1 x 10^7 cells/ml) on which a 930 V pulse voltage (BioRad: Gene Pulser II) was then applied. The resulting cell suspension was diluted 20-fold with DMEM medium and washed twice by centrifugation. The cells were suspended and plated to a glass-bottom dish (p35-0-14-C, MatTek, Ashland) for culturing.

4. Confirmation of Expression of c-fos mRNA

4-1. Dot blotting

[0103] Expression of c-fos mRNA in the Cos7 cells treated with the expression vector was confirmed by dot blotting. The cells were removed off by trypsin treatment after one day of plating, and washed with PBS. A QuickPrep Total RNA Extraction Kit (Pharmacia Biotech) was then used to extract the total RNA from the cells. The RNA solution extracted from the cells was transferred onto a nylon membrane and crosslinked thereto by ultraviolet irradiation (Bio-Rad: Gene Linker). This was allowed to react with a digoxigenin (DIG)-labeled c-fos RNA probe (2.1 kb RNA with a sequence complementary to c-fos mRNA). Then, after reacting with alkali phosphatase-conjugated anti-DIG antibody, a DIG Nucleic Acid Detection Kit (Boehringer Mannheim) was used to form 4-nitroblue tetrazolium which was catalyzed by alkalinephosphatase. The quantity of the c-fos RNA probes hybridizing to the nylon membrane was determined by quantifying the amount of the formed 4-nitroblue tetrazolium using a densitometer. c-fos RNA of known concentrations that was synthesized by *in vitro* transcription reactions was plotted in like manner, and on that basis the quantity of the c-fos mRNA contained in the RNA fractions that had been extracted from the cells was estimated. The DIG-labeled c-fos RNA probes were prepared by *in vitro* transcription reactions, in which a linearized pBluescript-cFos prepared by treatment of the plasmid with the restriction enzyme EcoRV was used as a target and T3 RNA polymerase was driven. The reactions were performed using a T7 MEGAscript kit (Ambion). The expression of c-fos mRNA was confirmed at 12, 24, 48, 72 and 96 h after transfection.

4-2. *in situ* hybridization

[0104] Fluorescence *in situ* hybridization (FISH) was performed to confirm the ratio of expressing cells. The FISH was carried out according to a known protocol. After Cos7 cells treated with pME18S-cFos were cultured for one day,

the cells were fixed for 15 min at room temperature with 4% paraformaldehyde/PBS (pH 7.4). The fixed cells were treated with the DIG-labeled c-fos RNA probe. The cells were then stained with the FITC-conjugated anti-DIG antibody and observed under a fluorescence microscope. It was confirmed that more than 80-90% of the transfected cells expressed c-fos mRNA.

5. Confirmation of Hybridization of Probes to c-fos mRNA in Living Cells by *in situ* Transcription

[0105] The following experiment was conducted to confirm that the fluorescently labeled probes (D2F, A2F) hybridize to c-fos mRNA in the c-fos mRNA-expressing cells when introduced therein. Specifically, the probes were introduced into the cells when they were alive, and the cells were then fixed. Reverse transcription was carried out using the introduced probes as primers in the fixed cells, and synthesis of cDNA was detected (*in situ* transcription method (IST method), Politz, J.C., Taneja, K.L., Singer, R.H. (1995), Nucleic Acids Research, 23, 4946-4953). Cos7 cells transfected with pME18S-cFos were cultured overnight. The fluorescently labeled probes and the transfection reagent TransFast (Promega) were mixed at a charge ratio of 1:1. The mixture, 10 μM (final concentration), was added to the cells which were pre-cultured in serum-free medium for one hour, and the cells were incubated at 37 °C for one hour. After washing the cells, they were treated with 4% paraformaldehyde/PBS (pH 7.4) at room temperature for 15 min to fix the cells. The cell membrane permeability was increased by treatment with 0.5% TritonX-100 (90 sec), and then the cells were immersed in a transcription reaction solution containing Moloney Murine Leukemia Virus Reverse Transcriptase (RNaseH-), 0.35 mM DIG-labeled dUTP and 1 mM dNTP and incubated at 30 °C for 1.5 h for reverse transcription reactions. The cells were then treated with the FITC-conjugated anti-DIG antibody and observed with a fluorescence microscope.

[0106] As a result, the transfected cells into which D2F had been introduced exhibited strong fluorescence in the whole cytoplasm. That is, c-fos cDNA had been synthesized in the cytoplasm. When D2F was introduced into Cos7 cells that had not been transfected, no fluorescence was observed in the cytoplasm. No fluorescence was observed in the cells when an oligo DNA with the same sequence as the 657-676 site of c-fos mRNA (sense probe, with a base sequence complementary to D2F) was allowed to act on the transfected cells. These results confirmed that D2F hybridizes to c-fos mRNA in living Cos7 cells that express c-fos mRNA due to transfection. Similar results were obtained for A2F, confirming that A2F also hybridizes to c-fos mRNA in Cos7 cells.

[0107] The base sequences of the sense probes used in the experiment were the following. The synthesis and purification were carried out by the method described above.

Oligo DNA with sequence of 657-676 site

5'-(Bodipy493/503)GCGGAGACAGACCAACTAGA-3'

Oligo DNA with sequence of 677-696 site

5'-AGATGAGAAGTCTGCT(Cy5)TTGC-3'

6. Change in Fluorescence Image Resulting from Hybrid Formation in Living Cells

[0108] A solution of D2FB/streptavidin as the donor probe and A2FB/streptavidin as the acceptor probe mixed in a molar ratio of 1:1 (probe concentration: 2×10^{-5} M, PBS buffer) was injected into Cos7 cells. A fluorescence microscope was used to take DD images (Bodipy493/503 fluorescence) and AA images (Cy5 fluorescence), as well as DA images (image of the Cy5 fluorescence by irradiation of light with the excitation wavelength of Bodipy493/503), in order to observe the FRET from Bodipy493/503 to Cy5. The DA images were observed using the following filters:

Excitation filter: BP450-490
Dichroic mirror: FT510
Barrier filter: BP660-710

[0109] When FRET occurs, the fluorescence intensity of the DD image (donor) decreases while the fluorescence intensity of the DA image (acceptor) increases. The ratio of the fluorescence intensities of the DA image and DD image (DA/DD) represents the efficiency of FRET. This was confirmed by the following experimental results. Here, when the

ratio of the DA and DD images was to be obtained, processing was performed to subtract background from the respective images.

[0110] Specifically, D2FB/streptavidin and A2FB/streptavidin were mixed in 1 x SSC solution at 1 x 10^{-5} M each (molar ratio of 1:1). To this was added a 40mer RNA with a base sequence complementary to the probes at ratios of 0.1, 0.2, 0.5, 0.75, 1.0 and 1.25, and the mixtures were allowed to stand at room temperature for 30 min. Each donor probe/acceptor probe/40mer RNA mixture was then dropped onto a cover slip and the fluorescence images (DD image, DA image) of each solution were taken. The ratio of the fluorescence intensity of the DD image and the fluorescence intensity of the DA image (DA/DD) was obtained, and was plotted against the molar ratio of the RNA and probes (RNA/probes) (Fig. 4).

[0111] Larger amount of the 40mer RNA was added, a decrease of fluorescence intensity in the DD image and an increase of fluorescence intensity in the DA image became larger. That is, the DA/DD value increases in response to increasing the amounts of hybrid formed among D2FB/streptavidin, A2FB/streptavidin and the 40mer RNA. The DA/DD value increased from 0.08 for the probes alone (RNA/probes = 0) to 1.5 for RNA/probes = 1.

[0112] The DA/DD value was 0.08 in the cytoplasm of the Cos7 cells immediately after injection of the D2FB/streptavidin and A2FB/streptavidin mixture, and this value was the same as the value for the injection solution. No change was observed in the DA/DD value after incubating the cells on the microscope stage for 15 min. The cells were then injected with a 40mer RNA (2 x 10^{-5} M, dissolved in DEPC-water) that had the same sequence as the 657-696 site of c-fos mRNA. The fluorescence image taken after 2 min showed a decrease in fluorescence intensity in the cytoplasm in the DD image and an increase in fluorescence intensity in the DA image (Figs. 5A-5D). The DA/DD value was 1.5. The DA/DD values plotted against time are shown in Fig. 7.

[0113] By referring to Figs. 5A-5D, it is understood that most of the donor probes and the acceptor probes in the cytoplasm formed 3-member hybrids with the 40mer RNA. When a 40mer DNA with the antisense sequence to the 657-696 site of c-fos mRNA (prepared by linking the donor probe base sequence with the acceptor probe base sequence continuously) was injected as a control experiment, no change was observed in the fluorescence images (Figs. 6A-6D). These results indicate that both D2FB/streptavidin and A2FB/streptavidin introduced into the cytoplasm can rapidly hybridize to the target RNA when it is present (i.e., the probes can freely diffuse in the cytoplasm) and that the fluorescence images change and, as the result, the DA/DD value increases with formation of a 3-member hybrid of the donor probe, acceptor probe and target RNA in the cytoplasm.

[0114] The base sequences of the RNA and DNA used in the experiment were the following, and the synthesis and purification were carried out by the method described above.

40mer RNA with same sequence as 657-696 site of c-fos mRNA

5'-GCGGAGACAGACCAACUAGAAGAUGAGAAGUCUGCUUUGC-3'

40mer DNA with same sequence as 657-696 site of c-fos mRNA

5'-GCGGAGACAGACCAACTAGAAGATGAGAAGTCTGCTTTGC-3'

40mer DNA with antisense sequence to 657-696 site of c-fos mRNA

5'-GCAAAGCAGACTTCTCATCTTCTAGTTGGTCTGTCTCCGC-3'

7. Detection of c-fos mRNA in Living Cells

[0115] A solution of mixtures of D2FB/streptavidin as the donor probe and A2FB/streptavidin as the acceptor probe

mixed at a molar ratio of 1:1 (probe concentration: 2 x 10^{-5} M, PBS buffer) was injected into Cos7 cells that had been transfected with pME18S-cFos (cells expressing c-fos mRNA).

[0116] No increase in the DA/DD value in the cytoplasm was observed during 20 min after injection (Figs. 8A-8D and Fig. 12). This result suggests that streptavidin produces steric hindrance to hybridization of the oligo DNA probes to the c-fos mRNA in the cytoplasm. Thus, an oligo DNA having a "linker" introduced between the oligo DNA for hybridization and streptavidin was used as a probe. Oligo DNA was used as the "linker"; 10 C (cytosine) was attached to the 3'-end of the donor probe, and 20 C was attached to the 5'-end of the acceptor probe.

[0117] The streptavidin-bound oligo DNA probes used in the experiment were the following, and the synthesis and purification of the oligo DNAs, and binding with streptavidin, were carried out in the manner described above.

Linker-introduced donor probe (D2FBL):

5'-

(Bodipy493/503)TCTAGTTGGTCTGTCTCCGCCCCCCCCCC(Biotin)-

3'-(streptavidin)

Linker-introduced acceptor probe (A2FBL):

(streptavidin)-5'-

(Biotin)CCCCCCCCCCCCCCCCCCCCGCAAAGCAGACTTCTC(Cy5)ATCT-

3'

[0118] D2FBL and A2FBL were mixed in a molar ratio of 1:1, and were injected into Cos7 cells transfected with pME18S-cFos (Cos cells expressing c-fos mRNA). Figs. 9A through 9D show the DD image, DA image, AA image and phase contrast (Ph) image taken 5 min after injection, respectively. The AA image shows that Cy5 fluorescence was observed throughout the whole cytoplasm, and the strong fluorescence was observed in a certain region near the nucleus. This indicates that while Cy5 (i.e., the acceptor probe A2FBL) is diffusely distributed throughout the whole cytoplasm, its concentration is higher in the certain regions near the nucleus. The DD image shows that Bodipy493/503 fluorescence was observed throughout the whole cytoplasm. Relatively strong Bodipy 493/503 fluorescence is also observed in the region near the nucleus similarly to the distribution of the C5 fluorescence, but not as clearly as in the AA image. The DA image presenting fluorescence due to FRET shows that quite strong fluorescence was observed in the same regions where strong fluorescence was observed in the DD image and AA image. These fluorescence images indicate that the donor probes and acceptor probes are present at higher concentrations in regions near the nucleus, and that FRET occurs with higher efficiency in these regions than in the other regions of the cytoplasm. Fig. 10A shows the DA/DD ratio image at 5 min after injection, which evaluates the FRET efficiency in the cells and is presented in pseudo-color. According to Fig. 10A, the DA/DD values were non-uniformly distributed throughout the cell. The DA/DD values in the regions near the nucleus were 0.7-1.2, and 0.3-0.4 in the other regions. These values were larger than the value for the injection solution (0.08), indicating that FRET had occurred in most of the cytoplasm. Fig. 10B shows the DA/DD ratio image at 20 min after injection in pseudo-color. The distribution pattern of fluorescence remains almost unchanged from Fig. 10A; the DA/DD value increased. This indicates that hybridization of the probes to the c-fos mRNA had been progressing during 20 min after injection. Figs. 11A through 11D are other examples of DA/DD images, and also exhibits changes in the fluorescence images with time. The DA/DD values increased in some regions of the cytoplasm 5 min after injection. Further, increasing DA/DD values were observed in the cytoplasm as time progressed. The DA/DD values in the nucleus, however, were still low values. Fig. 12 is a graph showing the average DA/DD values in the cytoplasm plotted against time after injection.

[0119] When the same pair of probes was injected into Cos7 cells that had not been transfected with pME18S-cFos (c-fos mRNA non-expressing cells) as a control experiment, the DA/DD values were approximately as low as the value for the injection solution (0.08) (Fig. 12, Figs. 13A-13D). When a mixture of the acceptor probe with the same base sequence as the 657-676 site of c-fos mRNA and the donor probe with the same base sequence as the 677-696 site thereof (probes with sense sequences, which probes contained the same linker portions as the antisense probes mentioned above) was injected into Cos7 cells transfected with pME18S-cFos (c-fos mRNA-expressing cells) as another control experiment, the DA/DD values were approximately as low as the value for the injection solution (0.08)

(Fig. 12, Figs. 14A-14D). Figs. 15A through 15D shows the DD image, DA image, AA image and phase contrast image of a cell taken 5 min after injection, respectively. The DD image and AA image show that both Bodipy493/503 fluorescence and Cy5 fluorescence was observed uniformly throughout the whole cytoplasm. In the DA image, little fluorescence was observed. Fig. 16A, similarly to Fig. 10A, shows the DA/DD ratio image at 5 min after injection in pseudo-color. The DA/DD values are approximately 0.1, viz., approximately the same value for the injection solution (0.08). Fig. 16B shows the DA/DD ratio image of another cell at 20 min after injection in pseudo-color. From this image it is seen that the distribution of fluorescence and the DA/DD values were unchanged during 20 min after injection. These results indicate that no FRET occurred, and therefore that no hybrids were formed in these control samples.

[0120] The streptavidin-bound oligo DNA probes used in the experiment were the following, and the synthesis and purification of the oligo DNAs, and binding with streptavidin, were carried out by the method described above.

Linker-introduced sense probes

With same base sequence as 657-676 site (D2SFBL):

5'-

(Bodipy493/503)AGATGAGAAGTCTGCTTTGCCCCCCCCCCCCCCCCCCC

C(Biotin)-3'-(streptavidin)

With same base sequence as 677-696 site (A2SFBL)

(streptavidin)-5'-(Biotin)CCCCCCCCCCGCGGAGACAGACCAAC

(Cy5)TAGA-3'

[0121] The above results indicate that hybridization with c-fos mRNA by the donor probe having the base sequence complementary to the 657-676 site of c-fos mRNA and the acceptor probe having the base sequence complementary to the 677-696 site of c-fos mRNA, which was introduced into the cells, was detectable as a fluorescence image under a microscope. The donor probe and acceptor probe with no linker portion form hybrids with c-fos RNA *in vitro* (in an aqueous solution). In cells, they also form hybrids with 40mer RNA having the complementary base sequence. Notwithstanding, when these probes were introduced into c-fos mRNA-expressing cells, no FRET was observed in the cytoplasm (no increase in the DA/DD values occurred), which suggests that the degree of steric hindrance by streptavidin to hybridization of the probes with c-fos mRNA is much greater intracellularly than in aqueous solution.

(Example 3) Estimation of the Quantity of c-fos mRNA Expression in Living Cells

[0122] In the same manner as Example 2, approximately 10^5 molecules of the linker-introduced donor probe (D2FBL) and the linker-introduced acceptor probe (A2FBL) were injected into individual Cos7 cells that had been transfected with pME18S-cFos, at 3-5, 24, 48 and 72 hours after transfection. The total fluorescence intensity of the cytoplasm for the DD image and the DA image were determined respectively, and the DA/DD values for the individual cells were estimated. In Fig. 17, the DA/DD value for each cell was plotted. The average value among the cells at each time was also shown. The DA/DD values were larger than 0.1, and there was large divergence between cells. As a control experiment, the probes were injected into Cos7 cells that had not been transfected with pME18S-cFos, in the same manner as Example 2. As another control experiment, probes with the sense sequence (D2SFBL and A2SFBL) were injected into Cos7 cells that had been transfected with pME18S-cFos. The injection was carried out 24 h after transfection. The DA/DD values for the control samples were approximately 0.1, which was almost the same as the value for the injection solution (0.08).

[0123] The average number of hybrid present in the cytoplasm per cell was calculated from the average DA/DD value. Here, the total number of probe molecules in the cell was assumed to be 10^5. As a result, values of approximately 13,000, 30,000, 24,000 and 19,000 were obtained for the injection experiments at 3-5, 24, 48 and 72 h after injection, respectively. These values approximately match the values determined by dot plotting the number of molecules of expressed c-fos mRNA per cell (Fig. 18). This method can therefore assay the expression of genes (mRNA) on the single cell level.

[0124] From the invention thus described, it will be obvious that the invention may be varied in many ways. Such

variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended for inclusion within the scope of the following claims.

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Claims

1. A method for detecting a target nucleic acid existing in cytoplasm of a living cell, the method comprising:

   introducing into the cytoplasm, a detection probe bound to a nuclear membrane unpermeable molecule via a linker and labeled with fluorescent dye(s), the probe having a base sequence capable of hybridizing to the target nucleic acid;
   forming a hybrid between the target nucleic acid and the probe; and
   determining any change in fluorescence of the fluorescent dye(s) due to formation of the hybrid.

2. The method according to claim 1, wherein the detection probe comprises a first probe member and a second probe member, the first and second probe members have base sequences capable of hybridizing to the target nucleic acid adjacently with each other, the first probe member is labeled with an energy donor fluorescent dye and the second probe member is labeled with an energy acceptor fluorescent dye, the change in fluorescence of the fluorescent dyes is fluorescence resonance energy transfer (FRET) from the energy donor fluorescent dye of the first probe member to the energy acceptor fluorescent dye of the second probe member.

3. The method according to claim 1 or 2, wherein the nuclear membrane unpermeable molecule is at least one member selected from the group consisting of proteins, sugars, beads, and metal particles.

4. The method according to any one of claims 1-3, wherein the detection probe further contain a nuclease-blocking molecule that is bound thereto via a linker.

5. The method according to claim 4, wherein the nuclease-blocking molecule is identical with the nuclear membrane unpermeable molecule.

6. The method according to any one of claims 1-5, wherein the detection probe is an oligonucleotide comprising from 10 to 20 bases.

7. The method according to any one of claims 1-6, wherein the target nucleic acid is mRNA.

Fig.1A

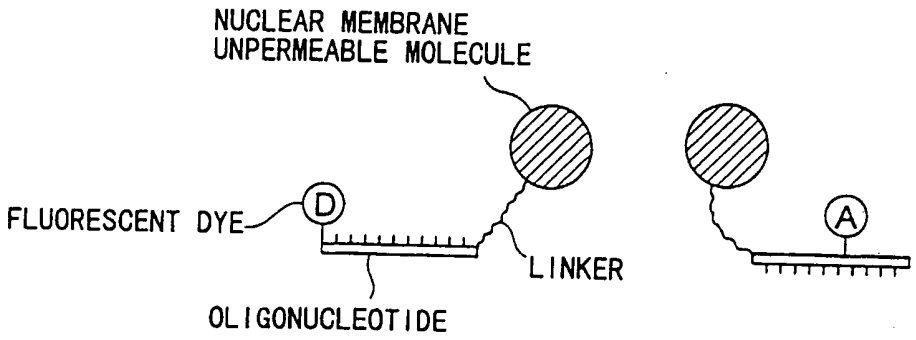

NUCLEAR MEMBRANE
UNPERMEABLE MOLECULE
FLUORESCENT DYE
D
LINKER
A
OLIGONUCLEOTIDE

Fig.1B

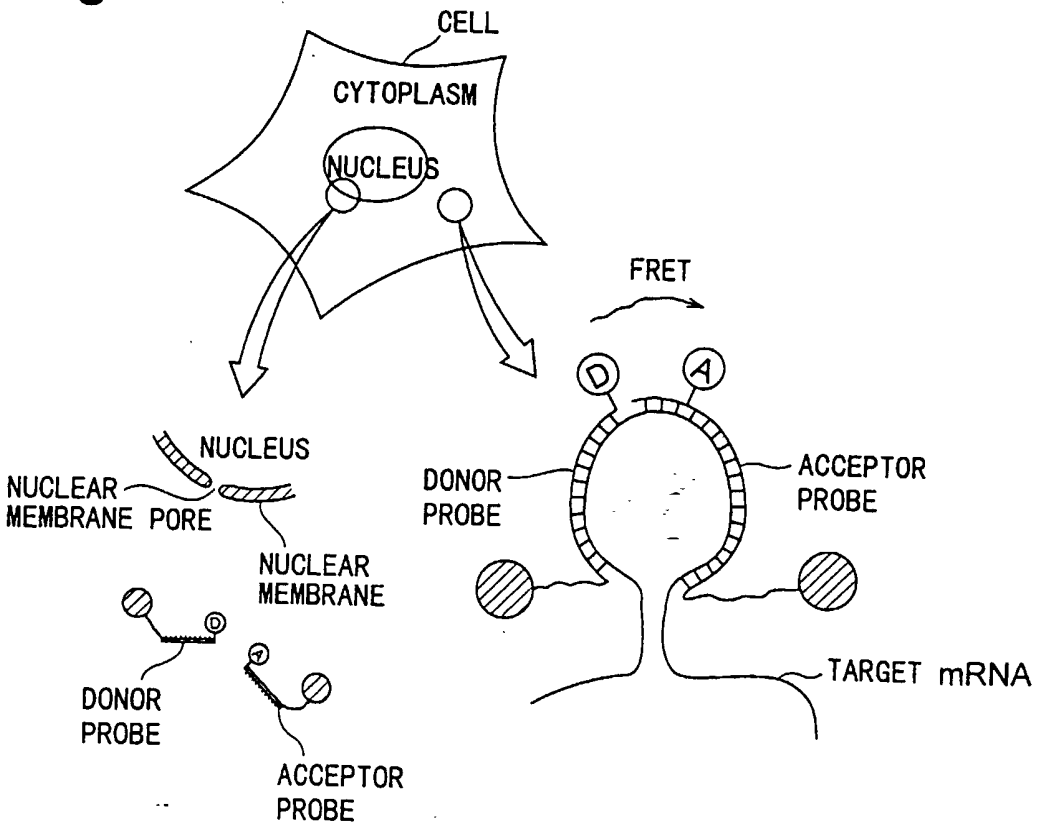

CELL
CYTOPLASM
NUCLEUS
FRET
D
A
NUCLEUS
NUCLEAR
MEMBRANE PORE
NUCLEAR
MEMBRANE
DONOR
PROBE
ACCEPTOR
PROBE
DONOR
PROBE
ACCEPTOR
PROBE
TARGET mRNA

Fig.2A

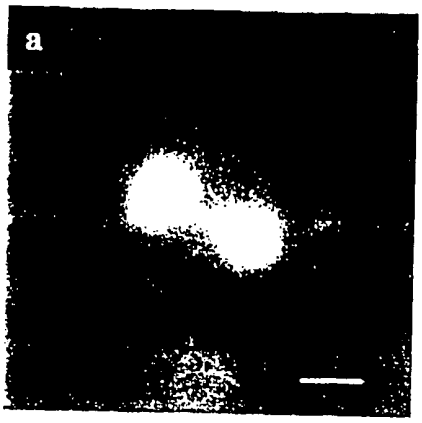


Fig.2B

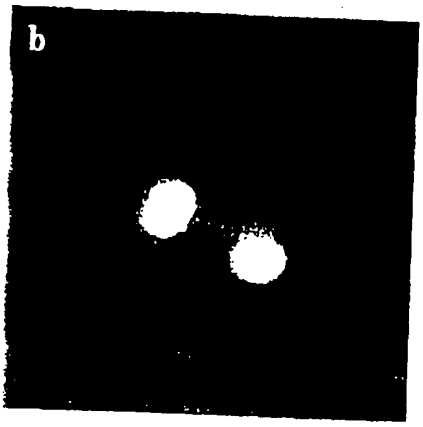


Fig.2C

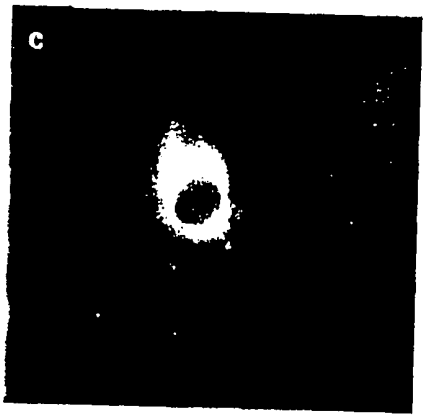
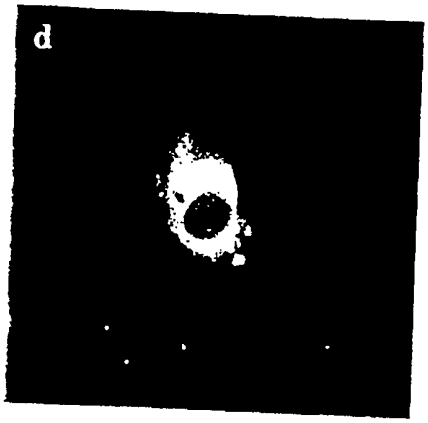


Fig.2D



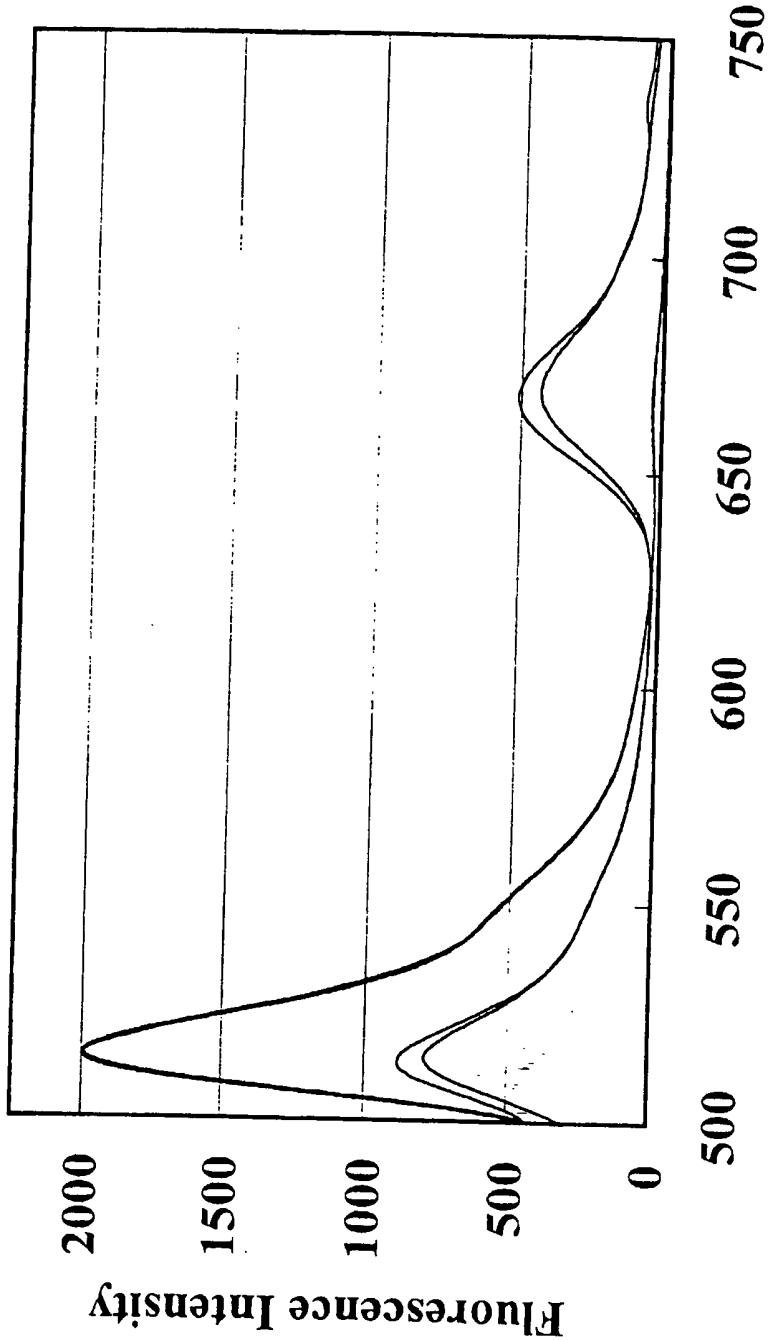

Fluorescence Intensity
2000
1500
1000
500
0
500
550
600
650
700
750
Wavelength (nm)

Fig.3

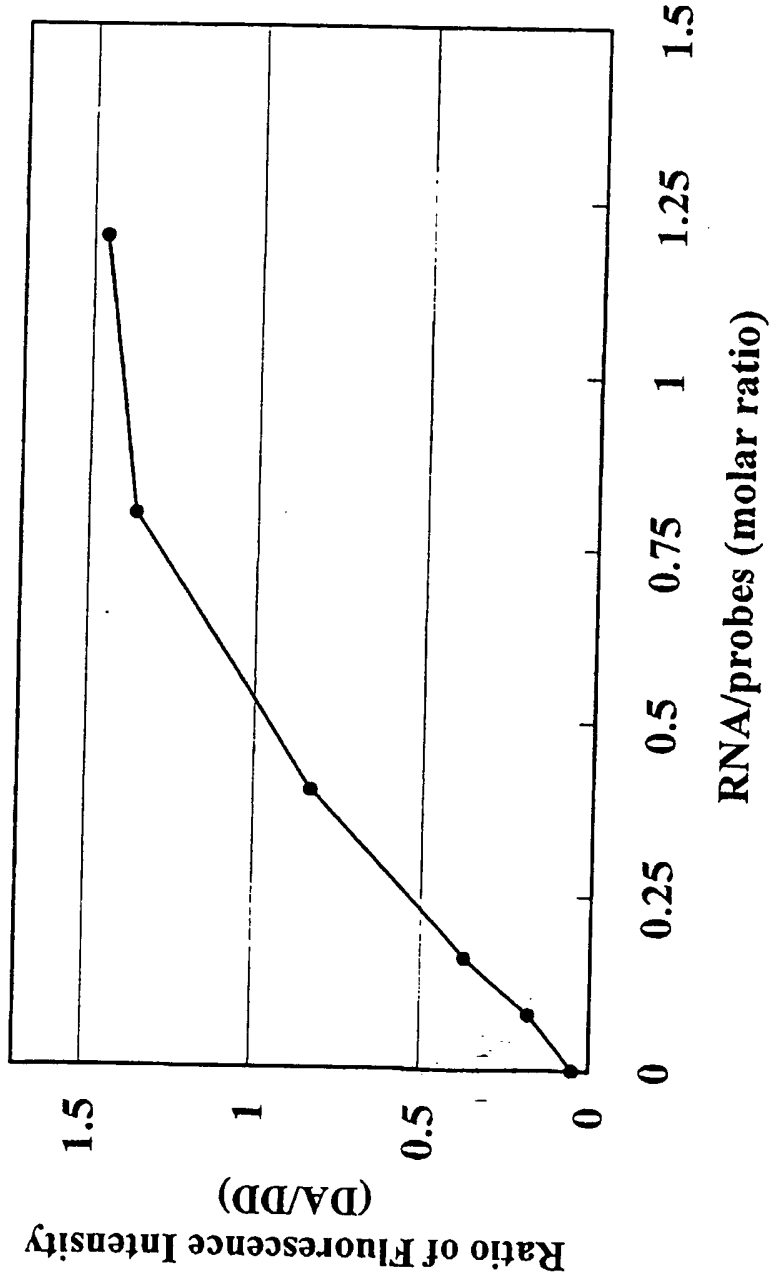

Ratio of Fluorescence Intensity
(DA/DD)
1.5
1
0.5
0
0
0.25
0.5
0.75
1
1.25
1.5
RNA/probes (molar ratio)

Fig.4

Fig.5A

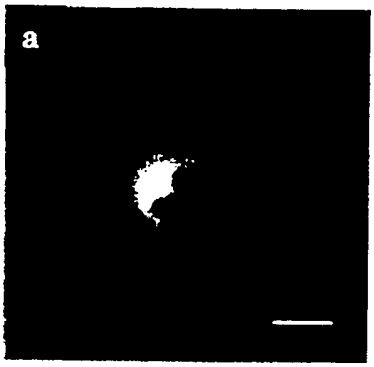


Fig.5B

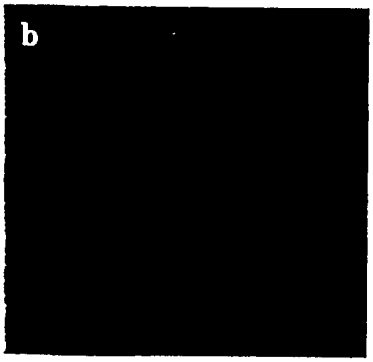


Fig.5C

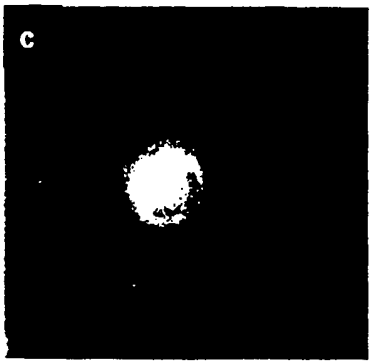


Fig.5D

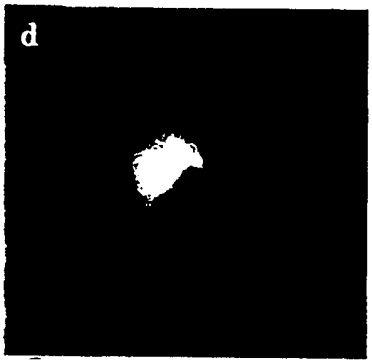


Fig.6A

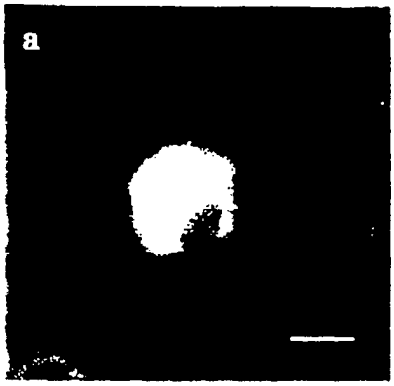


Fig.6C

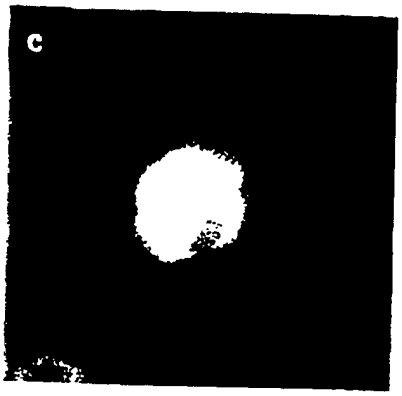


Fig.6B

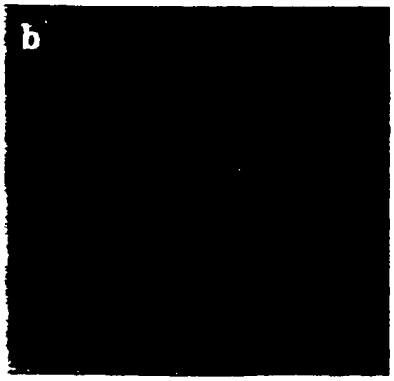
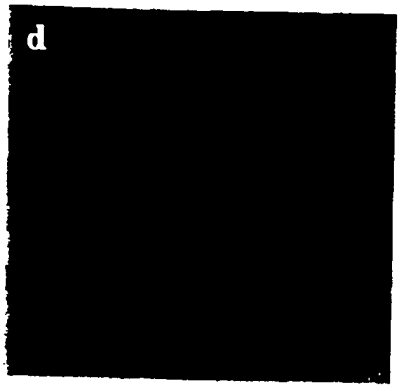


Fig.6D



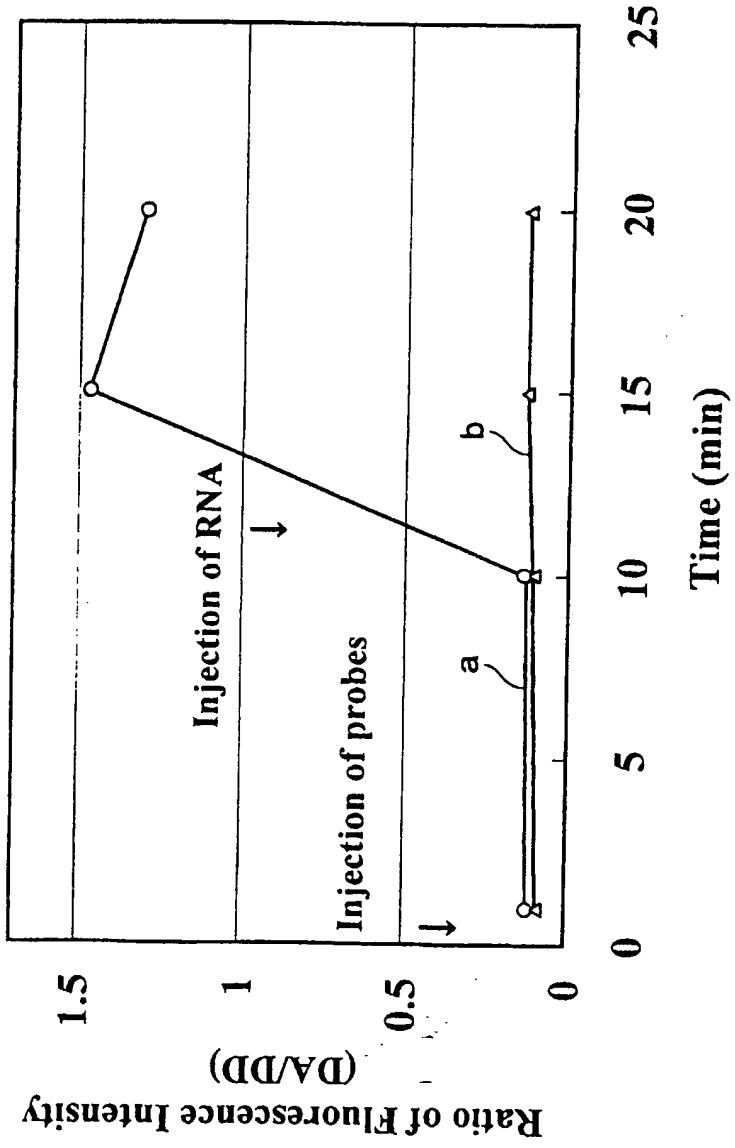

Ratio of Fluorescence Intensity
(DA/DD)
1.5
1
0.5
0
0
5
10
15
20
25
Time (min)
Injection of RNA
Injection of probes
a
b

Fig.7

Fig.8A

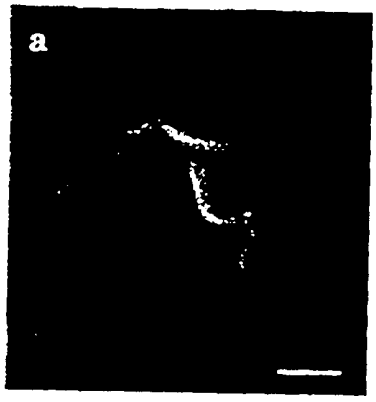


Fig.8B

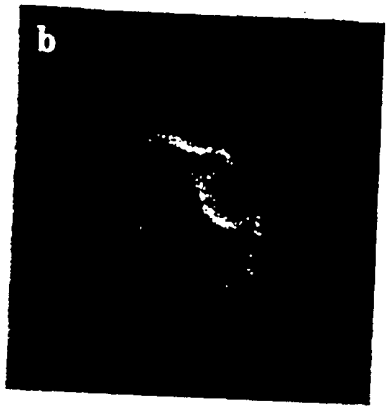


Fig.8C

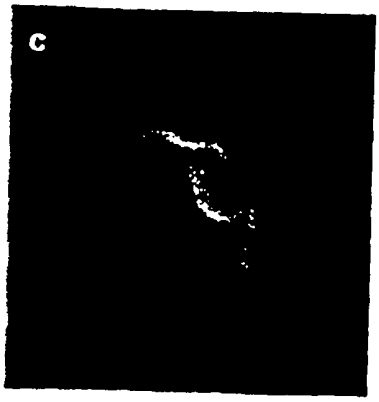
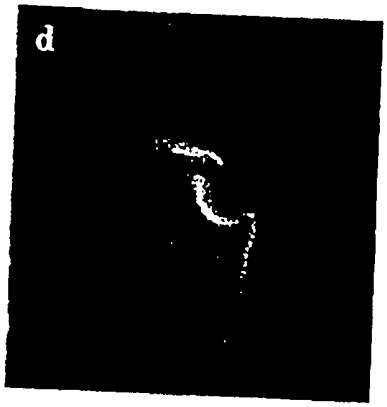


Fig.8D



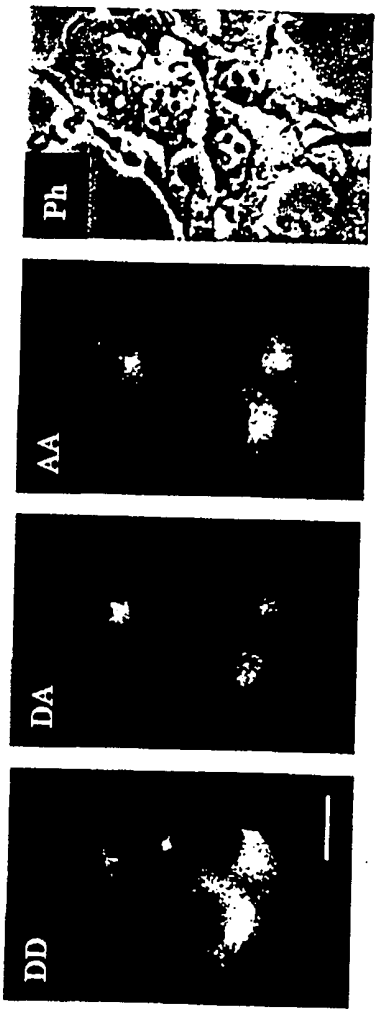


Fig.9A
DD
Fig.9B
DA
Fig.9C
AA
Fig.9D
Ph

Fig.10A

Fig.10B

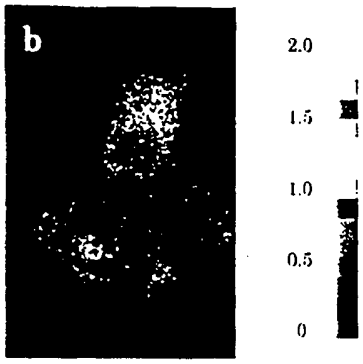


Fig.11A

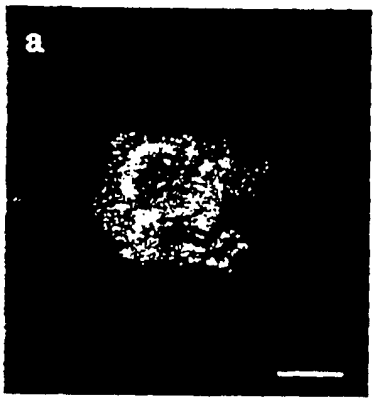


Fig.11B

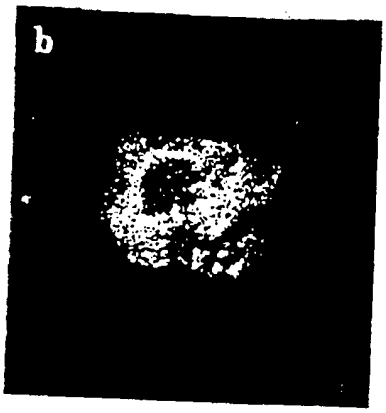


Fig.11C

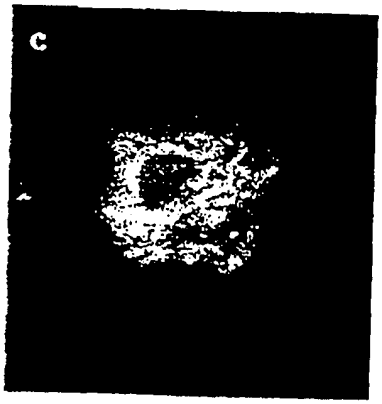


Fig.11D

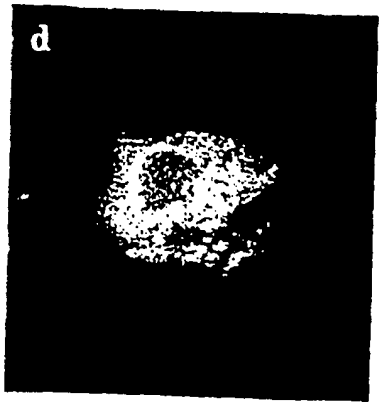


Fig.12

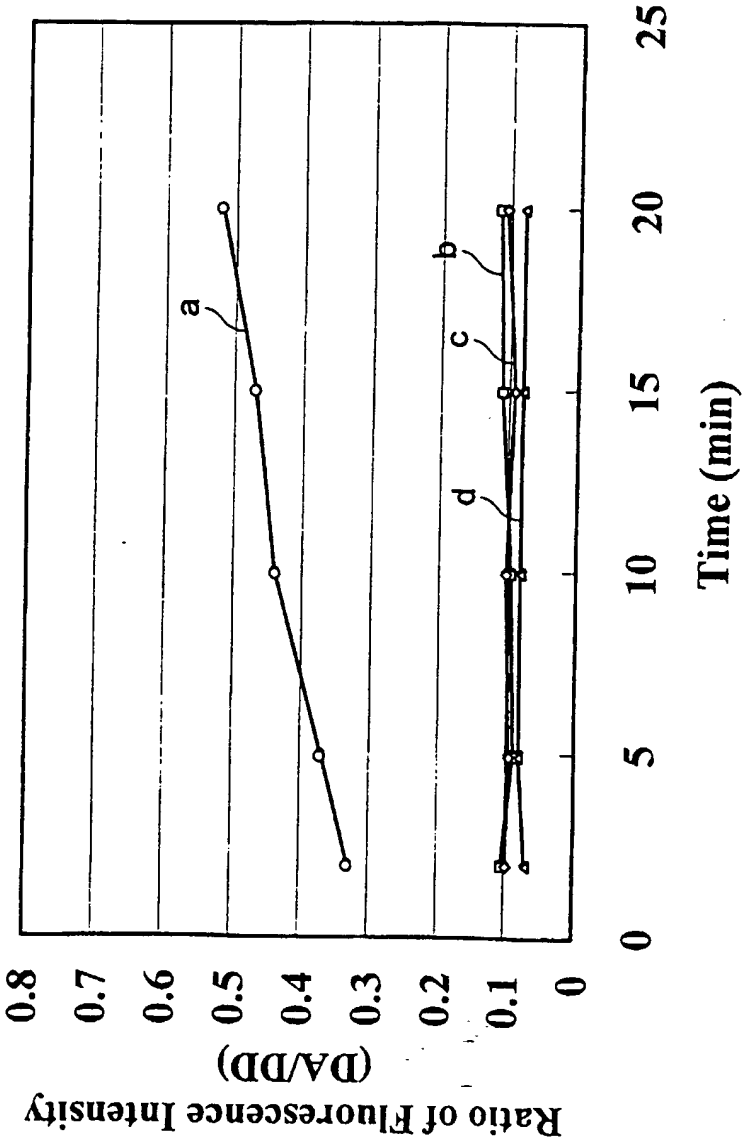

Ratio of Fluorescence Intensity
(DA/DD)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
Time (min)
a
b
c
d

Fig.13A

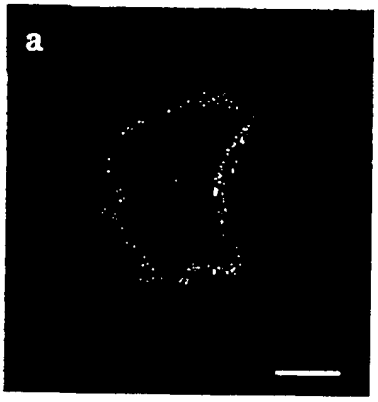


Fig.13B

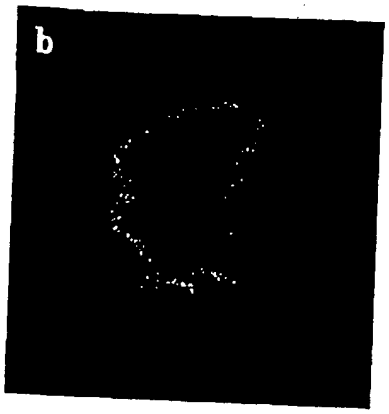


Fig.13C

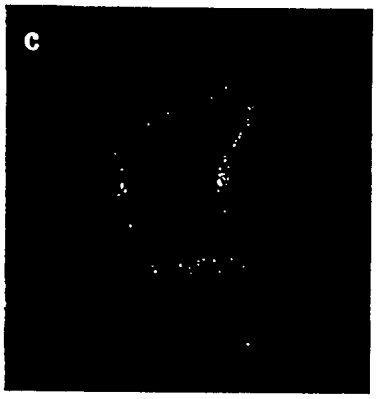


Fig.13D

Fig.14A

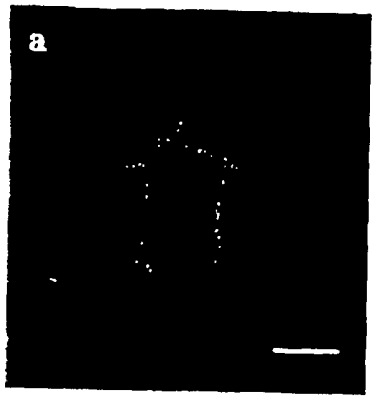


Fig.14B

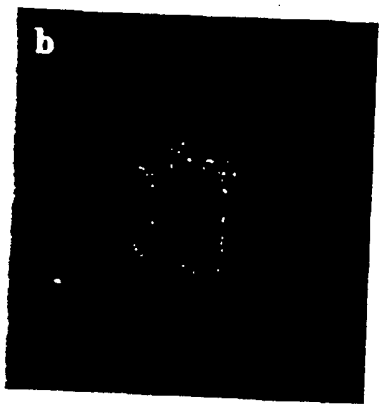


Fig.14C

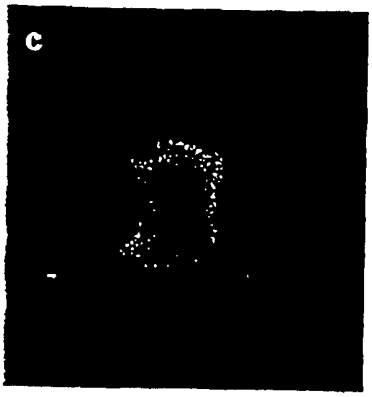
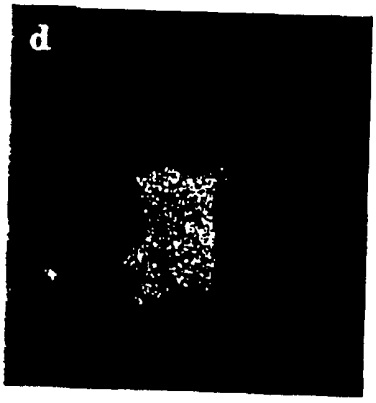


Fig.14D



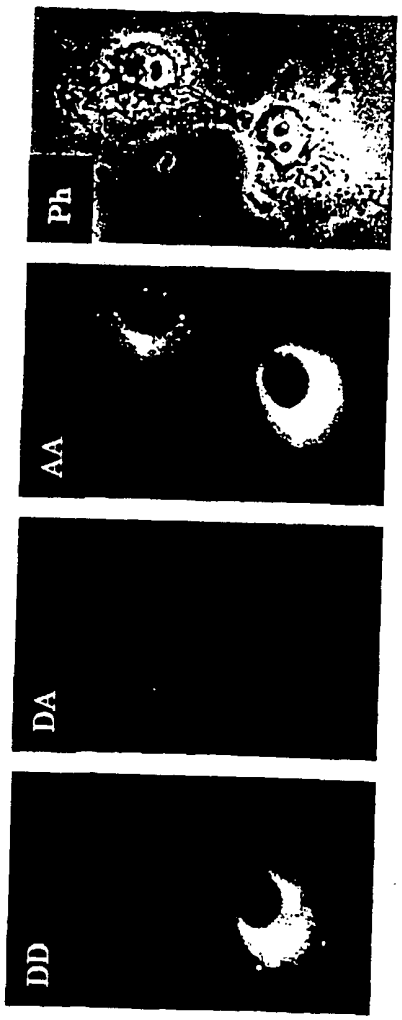


Fig.15A Fig.15B Fig.15C Fig.15D

Fig.16A

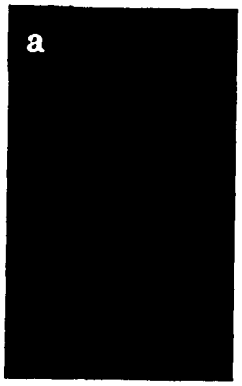
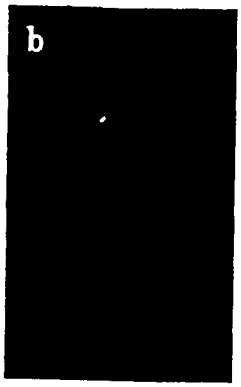


Fig.16B



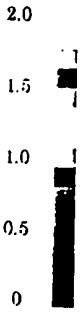
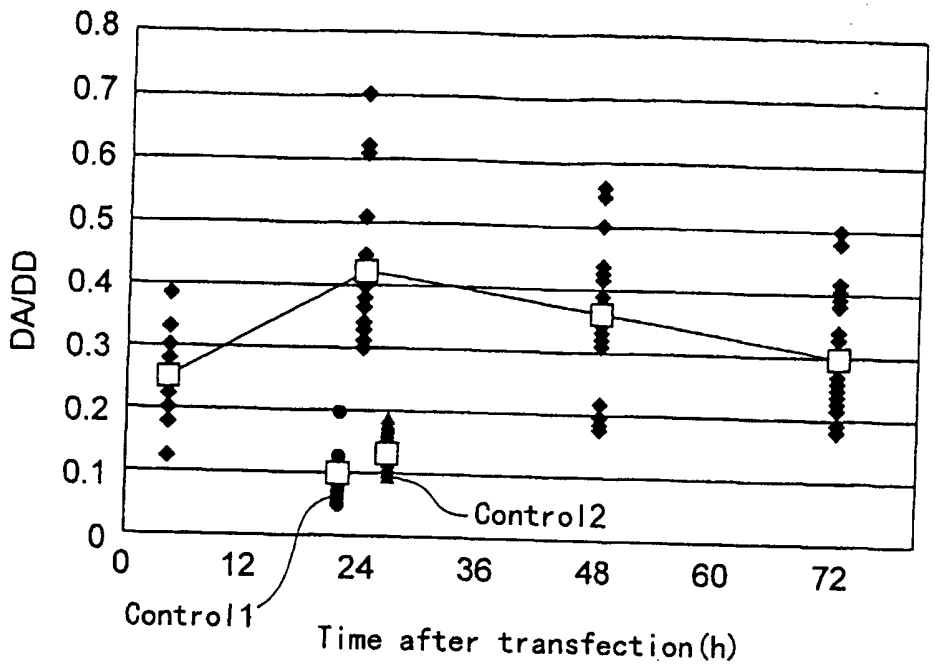


Fig.17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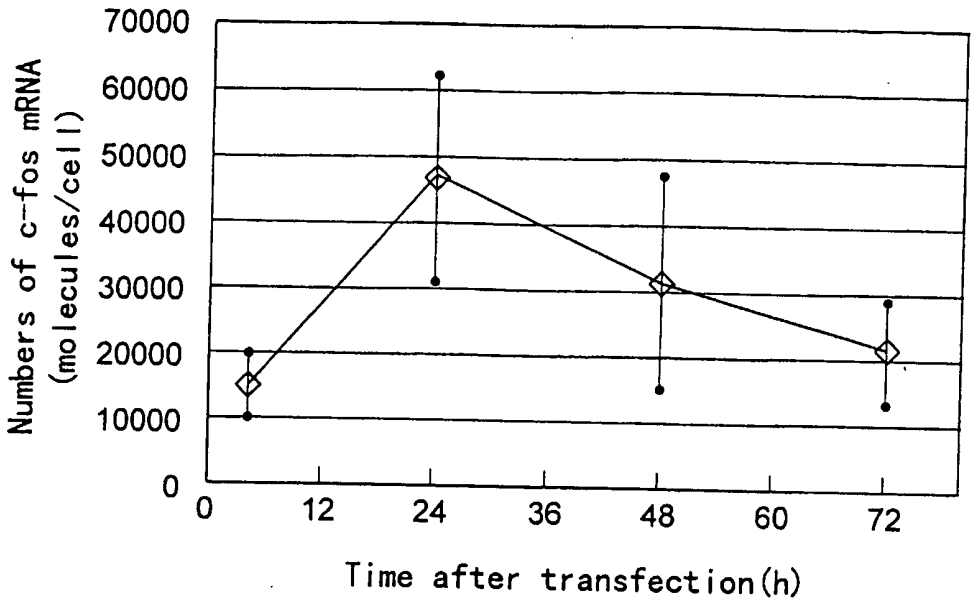
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DA/DD
0
12
24
36
48
60
72
Control1
Control2
Time after transfection(h)

Fig.18


70000
60000
50000
40000
30000
20000
10000
0
Numbers of c-fos mRNA (molecules/cell)
0
12
24
36
48
60
72
Time after transfection(h)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 12 6030

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	WO 98 13524 A (LAB OF MOLECULAR BIOPHOTONICS ;SUGA TAKAYUKI (JP); TSUJI AKIHIKO () 2 April 1998 (1998-04-02) see abstract	1,2	C12Q1/68 C12Q1/02 G01N33/50
A	WO 98 33897 A (LAB OF MOLECULAR BIOPHOTONICS ;IIDA YUKARI (JP); KONDO SATOSHI (JP) 6 August 1998 (1998-08-06) see abstract	1,2,6,7	
A	US 5 728 527 A (POLITZ JOAN C ET AL) 17 March 1998 (1998-03-17) * claims 1-3,9 *	1,2,6,7	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)
C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	30 March 2000	Osborne, H

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 99 12 6030

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

30-03-2000

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9813524 A	02-04-1998	AU 4321497 A	17-04-1998
		EP 0971038 A	12-01-2000
WO 9833897 A	06-08-1998	EP 0965635 A	22-12-1999
US 5728527 A	17-03-1998	AU 7401394 A	20-02-1995
		CA 2167804 A	02-02-1995
		EP 0712444 A	22-05-1996
		JP 9500280 T	14-01-1997
		WO 9503428 A	02-02-1995

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

VERIFICATION

The undersigned, of the below address, hereby certifies that he/she well knows both the English and Japanese languages, and that the attached is an accurate translation into the English language of the Certified Copy, filed for this application under 35 U.S.C. Section 119 and/or 365, of:

Application No.	Date Filed
2000-130793	April 28, 2000

The undersigned declares further that all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 24 th day of May, 2002

Signature: Shiro Terasaki

Name: SHIRO TERASAKI

Address: c/o SOEI PATENT AND LAW FIRM
Okura-Honkan, 6-12, Ginza 2-chome,
Chuo-ku,Tokyo 104-0061 Japan

(Document Name) Patent Application
(Reference Number) MBP-179
(Presentation Date) April 28, 2000
(Directly) Commissioner of the Patent Office
(IPC) C12N 15/00
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(Attorney)
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(Identification Number) 100092657
(Patent Attorney)
(Name) Shiro TERASAKI
(Priority Claimed)
(Application Number) 2000-028117
(Filing date) February 4, 2000
(Official Fee)
(Pre-Paid Master Note Number) 014708
(Amount to be paid) 21,000 yen
(Lists of the Article to be presented)
(Name of Article) Specification 1
(Name of Article) Drawing 1
(Name of Article) Abstract 1
(General Power of Attorney Number) 9804371
(Proof) Yes

[NAME OF DOCUMENT] Specification

[TITLE OF INVENTION] METHOD FOR SELECTIVELY SEPARATING LIVE CELLS EXPRESSING A SPECIFIC GENE

[WHAT IS CLAIMED IS]

[Claim 1]

A method for selectively separating live cells which have expressed mRNA comprising:

a first step of introducing a marker capable of labeling mRNA into cells in a live cell group containing live cells which have expressed a specific mRNA;

a second step of labeling said mRNA with said marker to obtain a live cell group containing live cells having the labeled mRNA; and

a third step of detecting said labeled mRNA to identify the live cells having said labeled mRNA and separating the identified live cells selectively from said live cell group obtained in said second step.

[Claim 2]

The method according to claim 1, wherein said marker in said first step is a probe which has a base sequence complementary to said mRNA and has been labeled with a fluorescent dye, said labeled mRNA in said second step is a hybrid of the probe and said mRNA, and the selective separation in said third step is performed by irradiating light to the live cell group containing live cells having the hybrid, identifying live cells which cause a change in fluorescence of said fluorescent dye based on formation of the hybrid, and separating the identified live cells from the live cell group.

[Claim 3]

The method according to claim 2, wherein said probe comprises a first probe and a second probe, the first probe and the second probe have base sequences capable of hybridizing to said mRNA adjacently, the first probe is labeled with an energy donor fluorescent dye and the second probe is labeled with an energy acceptor fluorescent dye, and said change in fluorescence is caused by fluorescence resonance energy transfer (FRET) from the energy donor fluorescent dye of the first probe to the energy acceptor fluorescent dye of the second probe.

[Claim 4]

The method according to claim 2 or 3, wherein the selective separation in said third step of said live cells based on the change in fluorescence is performed by a cell sorter (FACS).

[Claim 5]

The method according to any one of the claims 1 - 4, wherein said mRNA is an mRNA encoding a cytokine.

[Claim 6]

The method according to any one of the claims 3 - 5, wherein said mRNA is an mRNA encoding interleukin-2 (IL-2), said first probe is a probe having a base sequence set forth in SEQ ID NO: 9 in the Sequence Listing, and said second probe is a probe having a base sequence set forth in SEQ ID NO: 10 in the Sequence Listing.

[Claim 7]

The method according to any one of the claims 1 - 6, wherein the live cells selectively separated in said third step are T Helper 1 (TH1) cells.

[Claim 8]

The method according to any one of the claims 3 - 5, wherein said

mRNA is an mRNA encoding interleukin-4 (IL-4), said first probe is a probe having a base sequence set forth in SEQ ID NO: 17 in the Sequence Listing, and said second probe is a probe having a base sequence set forth in SEQ ID NO: 18 in the Sequence Listing.

[Claim 9]

The method according to any one of the claims 1 – 5 and 8, wherein the live cells selectively separated in said third step are T Helper 2 (TH2) cells.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[Technical Field to Which the Invention Belongs]

This invention relates to a method for selectively separating live cells which have expressed a specific gene.

[0002]

[Prior Art]

In the case that the translation products of a gene are cell surface molecules, a method for selectively separating cells which have expressed the specific gene, while being viable, is to make fluorescence labeled antibodies bind to the surface molecules for labeling the cells fluorescently, to identify fluorescing cells by flow cytometry, and to separate the identified cells with a cell sorter (Fluorescence Activated Cell Sorter, FACS). In addition, the panning method is also known wherein only the objective cells are absorbed on the bottom surface of a dish over which is covered with antibodies specifically binding to the cell surface molecules.

[0003]

In the case that the translation products of a gene are not cell surface molecules but localize in the cells (in the cytoplasm or in organella), the method described above cannot be adopted. In this case, it is theoretically possible to fluorescently label the gene-expressing cells by introduction of fluorescence-labeled antibodies that are specific to the molecules localized in the cells into the cells, through microinjection and to separate the objective gene-expressing cells with the cell sorter described above based on the difference in fluorescence intensity of the cells with irradiation of laser beam or the like.

[0004]

[Problems to be Solved by the Invention]

However, for the cell labeling method by microinjection described above, the method can not label many cells at once. the number of the cells to which the labeled antibodies can be introduced for one experiment is at most ten or less. In addition, it is not easy to introduce the solution of a polymer with the molecular weight greater than 120,000 like an antibody with high concentration into the cell because of its high viscosity. Therefore, microinjection is impractical to label the sufficient number of objective cells efficiently.

[0005]

In the case that the translation products of the gene are not cell surface molecules, but molecules that are liberated into the extracellular fluid and that do not remain in the cell or near the cell membrane, it is very difficult to selectively trap the molecules to separate the cells expressing the specific gene, from other molecules with the approaches described above. This is because, during the process where

polypeptide chains generated based on the genetic information are folded and secreted, their structure changes gradually and from time to time to prevent any known antibodies from binding to the polypeptide chains within or on the surfaces of live cells efficiently. Also, even in the case that the translation products are present on the surfaces of cells, it is difficult to selectively separate the cells unless the molecules are specifically present on the surfaces of particular cells.

[0006]

A typical example, in which there is a situation described above and it is difficult to separate the objective live cells selectively, includes the case where cells secreting a specific cytokine are selectively separated using the cytokine as a selection marker.

[0007]

When an antigen invades an organism, helper T cells (CD4+ T cells) that recognize the antigen as a foreign matter are activated, and then they will be differentiated into TH1 and TH2, which have different immune functions from each other: TH1 (T Helper 1) which is responsible for cellular immune functions, e.g., activation of macrophages to remove foreign matters by phagocytosis; and TH2 (T Helper 2) which has humoral immune functions, e.g., activation of B cells to produce antibody molecules to neutralize foreign matters and (See Fig. 94). TH1 and TH2 produce cytokines, interleukin-2 (IL-2) and interleukin-4 (IL-4), respectively. In the healthy state, TH1 and TH2 control their functions each other and keep a balance. However, once this relationship is disrupted, it causes various infections or autoimmune disorders.

[0008]

If TH1 or TH2 can be selectively separated and obtained, it will be medically important, because their application can be contemplated in supplementing immune functions or the like. Thus, a variety of attempts have been made to find molecules that are present on the surface of TH1 or TH2, that can be used for their separation and obtaining.

[0009]

For example, it has been reported that the tissue infiltration which is dependent on adhesive molecules, P- and E-selectin, is observed specifically with human TH1 (Austrup, F. et al. Nature, 385,81-83, 1997). This suggests that ligands adhering specifically to the selectins are present on TH1 cell surfaces. However, when reactivity for P- and E-selectin is examined by flow cytometry, the results are TH1:TH2=131:52 for P-selectin, and TH1:TH2=668:88 for E-selectin; therefore, the specificity is not complete. These results can be interpreted as reflecting the fact that particularly notable P- and E-selectin ligand expression is induced in TH1 under physiological conditions unique inflamed tissues (such as skin and joints).

[0010]

Receptors for CC-chemokine (CCR3), eotaxin, have also been reported to be present with approximate specificity on human TH2 cell surfaces (Sallusto, F. et al. Science, 277, 1997). However, since CCR3-negative T cell groups also include IL-4 producing TH2 cells in a proportion of 1.9%, the specificity is not complete. Furthermore, the presence of many more of the same receptors on eosinophil and basophil cell surfaces than on TH2 raises the risk of possible contamination by cells

other than TH2 if CCR3+ cells are simply separated from T lymphocytes that have been crudely purified from blood.

[0011]

The receptor CCR5 for other CC-chemokines such as MIP-1ß and IP10 and the receptor CXCR3 for the CXC-chemokine SDF-1 have been reported to be present with approximate specificity on human TH1 cell surfaces (Loestscher, P. et al., Nature, 391, 344, 1998). However, since one of the nine TH2 clones obtained here was CCR5+, the specificity is not complete. Furthermore, while TH1 shows higher CXCR3 gene expression and CXC-chemokine dependent migration than TH2, CXCR3 gene expression was also confirmed in all of the TH2 clones examined, and therefore, the specificity is not complete. Moreover, CCR5 and CXCR3 are also present on neutrophil cell surfaces, and therefore the risk exists of possible contamination by neutrophils in CCR5+ or CXCR3+ cells separated from T lymphocytes that have been crudely purified from blood.

[0012]

In addition, IL-12 (interleukin-12) receptor (IL-12R) has been reported to be present with approximate specificity on human TH1 cell surfaces (Rogge, L. et al., J. Exp. Med., 185, 825, 1997). However, while TH1 cell surfaces bear high affinity receptors (Kd value = 27 pM) and low affinity receptors (Kd value = 5 nM) for IL-12 at 140 molecules and 450 molecules per cell surface, respectively, similar low affinity receptors (Kd value = 2 nM) are also present on TH2 cells at 200 molecules per cell surface. This means that IL-12R cannot be used as a definitive TH1 cell surface marker. Moreover, since IL-12R is also present on the cell surfaces of NK cells, the risk exists of possible contamination by NK cells

in IL-12R positive cells separated from T lymphocytes that have been crudely purified from blood.

[0013]

IL-18 (interleukin-18) receptor (IL-18R) is another receptor reported to be present specifically on the cell surface of a TH1 clone established from transgenic mice with T cell receptors for ovalbumin (Xu, D. et al., J. Exp. Med., 188, 1485, 1998). However, like IL-18R, the ST2L molecule belonging to the interleukin-1 receptor (IL-1R) family is also known to be present on TH2 cell surfaces. Because gene homology within the IL-1R family is particularly high in humans, IL-18R cannot be considered a definitive cell surface marker in humans and no reports have yet been published on their presence specifically on TH1 cell surfaces. Also, since IL-18R is much more abundantly present on monocyte, neutrophil and NK cell surfaces than on TH1, the risk exists of possible contamination by cells other than TH1 in IL-18R positive cells separated from T lymphocytes that have been crudely purified from blood.

[0014]

The reports cited above suggested that receptors for cytokines, chemokines and the like present on TH1 or TH2 cell surfaces vary considerably in terms of amount (number of per cell surface) and quality (affinity of the receptors for their ligands or intracellular transduction of stimuli upon binding to ligands), and that the distribution of such receptors therefore highly favors either TH1 or TH2. The reason for the favorableness of cell surface molecules toward either TH1 or TH2 is believed to arise from the biological environment (physiological conditions) surrounding the helper T cells.

[0015]

For example IL-12, which is one of the ligands for these cytokine receptors, is a cytokine secreted by macrophages, etc. upon initial infection (Walker, W. et al., J. Immunol., 162, 5894, 1999), and IL-18 is also known to be produced by activated macrophages and Kupffer cells (Yoshimoto, T. et al., J. Immunol., 161, 3400, 1998). Naturally, both of these cytokines have more connections with TH1 than with TH2, in light of the cellular immunity function of the former, and they are considered to be factors that perform transduction of physiological information from macrophages to TH1 (i.e., that activate TH1 in the body).

[0016]

This suggests a connection between activation of macrophages by TH1 and reception of stimuli (IL-12 and IL-18) returned from macrophages, whereby macrophages activated by TH1 eliminate foreign matters in the body while also activating TH1. As this interdependent relationship is established at sites of inflammation in the body, it is fully expected for the number of receptors for IL-12 and IL-18 and the activity of the receptor molecules to increase significantly on TH1 cell surfaces. Further, since TH2 cells are not exposed to the same conditions in the body as TH1, it is surmised that they have no need to receive IL-12 or IL-18. However, as long as IL-12 or IL-18 receptors are detected even slightly on TH2 cell surfaces, it cannot be denied that TH2 also has the potential to respond to IL-12 or IL-18.

[0017]

It is therefore inconceivable that these cytokine receptors are definitive markers that can distinguish TH1 from among TH1 and TH2. In

addition, since these chemokine and cytokine receptors that are predominantly distributed on TH1 and TH2 cells are also found distributed among other cell types such as NK cells, they are considered impractical as markers for distinguishing TH1 or TH2 from each other in blood samples. For example, cell specimens containing CD4+ cells (helper T cells) that are separated and purified by common methods from blood samples taken from humans usually include contamination by monocytes and granulocytes, and these false positive cells may be expected to be mistaken for TH1 or TH2.

[0018]

As stated above, it is, therefore, very difficult to selectively separate TH1 and TH2 cells based on surface molecules. Moreover, since the cytokines (IL-2 and IL-4) produced by TH1 and TH2 do not remain in the cell or near the cell membrane but are liberated into the extracellular fluid, it is difficult to selectively separate TH1 and TH2 using these cytokines.

[0019]

The present invention has been made in consideration of the above-stated problems in the prior art. It is an object of the present invention to provide a separation method which allows one to selectively separate and obtain the objective cells, that is, the cells which have expressed a specific gene, when there are no cell surface molecules usable as markers in the cell, or when the cell surface molecules cannot be distinguished from each other even if they are present in the cell, or even when the molecules to be the markers are liberated into the extracellular fluid.

[0020]

[Means for Solving the Problems]

The present inventors have found that the problems in the prior art as described above result from the fact that the translation products of a specific gene (polypeptide) are used as targets (or markers) to separate gene-expressed cells. Based on this finding, the inventors pursued further research, and as a result, have found that if mRNA is used as a target (marker) which exists mainly on float in the cytoplasm and which is a transcriptional product of a gene instead of using the translation product (polypeptide) as a marker, it is possible to selectively separate the cells which have expressed a specific gene, while being viable, when there are no cell surface molecules usable as markers in the cell, or when the cell surface molecules are not ones which are strictly specific for the objective cells, or even when the molecules to be the markers are liberated into the extracellular fluid. The present invention has thus been accomplished.

[0021]

Specifically, the present invention provides a method for selectively separating live cells which have expressed a specific gene comprising:

a first step of introducing a marker capable of labeling mRNA into cells in a live cell group containing live cells which have expressed a specific mRNA;

a second step of labeling said mRNA with said marker to obtain a live cell group containing live cells having the labeled mRNA; and

a third step of detecting said labeled mRNA to identify the live cells having said labeled mRNA and separating the identified live cells selectively from said live cell group obtained in said second step.

[0022]

In the method for selectively separating live cells which have expressed a specific gene according to the present invention, it is preferable that said marker in said first step is a probe which has a base sequence complementary to said mRNA and has been labeled with a fluorescent dye, said labeled mRNA in said second step is a hybrid of the probe and said mRNA, and the selective separation in said third step is performed by irradiating light to the live cell group containing live cells having the hybrid, identifying live cells which cause a change in fluorescence of said fluorescent dye based on formation of the hybrid, and separating the identified live cells from the live cell group.

[0023]

It is also preferable that said probe comprises a first probe and a second probe, the first probe and the second probe have base sequences capable of hybridizing to said mRNA adjacently, the first probe is labeled with an energy donor fluorescent dye and the second probe is labeled with an energy acceptor fluorescence dye, and said change in fluorescence is caused by fluorescence resonance energy transfer (FRET) from the energy donor fluorescence dye of the first probe to the energy acceptor fluorescence dye of the second probe.

[0024]

In addition, in the method for selectively separating live cells which have expressed a specific gene according to the present invention, it is preferable that the selective separation in said third step of said live cells based on the changes in fluorescence is performed by a cell sorter (FACS).

[0025]

It is also preferable that said mRNA is an mRNA encoding a cytokine. It is more preferable that said mRNA is an mRNA encoding interleukin-2 (IL-2) and said first probe is a probe having a base sequence set forth in SEQ ID NO: 9 in the Sequence Listing and further that said second probe is a probe having a base sequence set forth in SEQ ID NO: 10 in the Sequence Listing.

[0026]

It is also preferable that said mRNA is an mRNA encoding interleukin-4 (IL-4) and said first probe is a probe having a base sequence set forth in SEQ ID NO: 17 in the Sequence Listing and further that said second probe is a probe having a base sequence set forth in SEQ ID NO: 18 in the Sequence Listing.

[0027]

In the present invention, it is preferable that the live cells selectively separated in said third step are T Helper 1 (TH1) or T Helper 2 (TH2) cells.

[0028]

[Specific Modes of the Invention]

The method for selectively separating live cells which have expressed a specific gene according to the present invention comprises:

a first step of introducing a marker capable of labeling mRNA into cells in a live cell group containing live cells which have expressed a specific gene;

a second step of labeling said mRNA with said marker to obtain a live cell group containing live cells having the labeled mRNA; and

a third step of detecting said labeled mRNA to identify the live cells having the labeled mRNA and separating the identified live cells selectively from the said live cell group obtained in the said second step.

[0029]

Markers to be introduced into the cells in the present invention may be those which can label mRNA and are not particularly limited. The marker produces labeled mRNA when it binds to mRNA in the cell. When mRNA does not exist in the cell, or when the marker is excessively introduced even when mRNA is present, the marker which does not involve a bond with mRNA may remain in the cell. Then, the markers are preferably detectable only when they have been bound to mRNA, or are those which can be detected to determine whether they have bound to mRNA or not.

[0030]

In the present invention, probes which have base sequences complementary to mRNA and which are labeled with a fluorescent dye (hereinafter called "fluorescence-labeled probe" in some cases) are preferably used as markers. These probes form hybrid with the mRNA in the cell. However, there are some probes which do not form hybrids in the cell, and it is necessary to detect the probes forming hybrids selectively as described above. Then, it is preferable to use, as the present probes, those which cause fluorescence changes based on the formation of hybrids.

[0031]

For these probes described above, two kinds of probes labeled with different fluorescent dyes from each other are used as a pair. In other

words, it is preferred to use a probe comprising a first probe and a second probe: the first probe and the second probe have base sequences hybridizable with the mRNA adjacently; the first probe is labeled with an energy donor fluorescent dye, and the second probe is labeled with an energy acceptor fluorescent dye.

[0032]

When the energy donor fluorescent dye which labels the first probe and the energy acceptor fluorescent dye which labels the second probe are brought close to each other at a proper distance (for example, less than 8nm), fluorescence resonance energy transfer (FRET) will be possible. Therefore, two fluorescent dyes in probe should be preferably placed at a distance which allows FRET to occur between the energy donor fluorescent dye and the energy acceptor fluorescent dyes when the three molecules, the first probe, the second probe and the mRNA form a hybrid. The preferable distance between two types of fluorescent dye in the formed hybrid depends on the kinds of fluorescent dye and sites of hybridization on mRNA. However, the distance between the two fluorescent dyes is preferably within 20 bases or less, more preferably within 2-4 bases. When probes which can generate FRET are designed, for example, Lakowicz, J. R. "Principles of Fluorescence spectroscopy" (1983), Plenum press, New York may be referred to.

[0033]

Energy donor fluorescent dyes which may be used in the present invention include 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-propionic acid and derivatives thereof (for example, Bodipy 493/503 or Bodipy FL available from Molecular Probes);

tetramethylrhodamine-5-(and-6)isothiocyanate) (TRITC) and derivatives thereof (available from Molecular Probes).

[0034]

Energy acceptor fluorescent dyes which may be used in the present invention include 1,1'-bis(ε -carboxypenty1)-3,3,3',3' -tetramethyl indodicarbocyanine-5,5' -disulfonate potassium salt and derivatives thereof (for example, Cy3 or Cy5, available from Amersham Pharmacia BioTech). X-rhodamine-5-(and-6)-isothiocyanate (XRITC) and derivatives thereof (available from Molecular Probes); 6-(((4,4-difluoro-5-(2-thieny1)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid and derivatives thereof (for example, Bodipy 630/650 or Bodipy 650/665, available from Molecular Probes).

[0035]

In the present invention, it is preferable to use Bodipy 493/503 as an energy donor fluorescent dye, and Cy5 or XRITC as an energy acceptor fluorescent dye.

[0036]

In the present invention, the number of bases of oligonucleotide to form a probe is not strictly limited. When the number of bases is extremely small, e.g., less than 10, it will, however, likely be difficult to form a fully stable hybrid. When the number of bases in a probe is large exceeding 50, not only the synthesis of the probe is difficult, but also the stability of the probe is degraded; and it can take a longer time to form a hybrid.

[0037]

The number of bases in a probe for hybridization is determined under the consideration of the conditions of hybridization, such as the concentration of target mRNA in a live cell to be used and the temperature in hybridization. Generally, the melting point of a hybrid formed with a probe and mRNA is elevated with the increased number of bases in a probe. For example, if the number of bases in a probe is 15 or so, a hybrid is likely to be formed at room temperature with adequately high efficiency, but at 37 °C, the hybrid is not to be formed with high efficiency. In order to detect a hybrid at 37 °C, it is desirable to use probes with the length of 15 bases or more, preferably 20 bases or more.

[0038]

On the other hand, the ratio of hybrid formation decreases as the number of bases increases when the number of bases of a probe is in the range of 15-20. For example, at room temperature, the time necessary to complete the hybridization between a probe of 20 bases and mRNA is several times longer than the time required for a probe of 15 bases.

[0039]

Taken these requirements together, it is further preferable that the number of bases in a probe is 10-50, more preferably 15-20.

[0040]

When the probes are designed, it is also important what site in the mRNA probes hybridize to, as well as the number of bases in a as described above. That is, mRNA itself is a molecule with complicated secondary and tertiary structure. Thus, even if the probe to be used has a base sequence complementary to a particular site of the mRNA, an obstruction for the probe to hybridize to the site often occurs in the

secondary and tertiary structure when the site interacts with other sites of the mRNA. In the present invention, therefore, the sites where the probes hybridize to have to be selected.

[0041]

The sites for hybridization are determined, for example, by approaches described below. First, base sequences of the objective mRNA are obtained from a database. If databases are not available, the base sequences of the mRNA may be determined by well-known methods. According to the information, a secondary structure of the mRNA is simulated. For this simulation, it is possible to use commercially available computer programs for predicting the secondary structure of RNA, such as DNAsis (Hitachi Software Engineering Inc.). Using the obtained secondary structure, a site with appropriate number of bases, which include the base sequences in the site free from obstruction for hybridization, is selected; an oligonucleotide having a base sequence complementary to the selected base sequence is synthesized; the synthesized oligonucleotide is fluorescently labeled; and the oligonucleotide is used as a probe.

[0042]

After several objective sites having 30-40 bases for hybridization are selected, it is preferable that each site is subdivided into two parts (each 15-20 bases). Oligonucleotides having base sequences respectively complementary to the subdivided parts are synthesized, and then fluorescently labeled to prepare the first probe and the second probe for use.

[0043]

In order to select a desirable set of probes from several sets of

probes selected and synthesized as above, the following method can be used: the first probe and the second probe are mixed and the fluorescence spectra is measured; then, the objective mRNA is added to the mixed solution to observe any changes in the fluorescence spectra. When the first probe, the second probe and mRNA is formed into a hybrid, FRET occurs between two types of fluorescent dyes; as a result, the fluorescence intensity of the energy donor dye decreases, while a fluorescence spectrum is obtained where the fluorescence intensity of the energy acceptor dye is increased. The aforementioned operation is carried out on several sets of first and second probes; the changes in fluorescence spectra are compared; and a set of probes with a greater change is selected. The objective mRNA used for these methods can be synthesized by in vitro transcription reaction using a recombinant plasmid DNA which includes cDNA corresponding to the mRNA.

[0044]

Then, hybrids and free probes are separated from each other using high performance liquid chromatography (HPLC) or the like to correctly evaluate the efficiency of each probe to hybridize to the objective mRNA, each probe and each objective RNA are mixed and reacted in an aqueous solution.

[0045]

The method to design the desirable fluorescence-labeled probes adopted in the present invention is described above in detail. Such fluorescence-labeled probe is one of the preferable forms of markers to be used in the present invention. In the present invention, after markers have been prepared, they may be introduced into the live cells which have

expressed a specific mRNA. As there are no limitations to the methods for introducing markers into the live cells, well-known methods are available, including microinjection, electroporation, and lipofection methods. In the present invention, the electroporation method is preferable, because the method can introduce markers into more than 1,000,000 of live cells in a short time at once.

[0046]

After the markers have been introduced into the live cells, mRNA is labeled with the markers in the cells. Fluorescence-labeled probes are used as markers, which specifically hybridize to the corresponding mRNA. The conditions for hybridization are not limited specifically, but, for example, live cells which have been introduced fluorescent labeled probes, may be retained at room temperature at least for a few minutes.

[0047]

After the cellular mRNA is labeled, live cells containing the labeled mRNA are identified by detecting the labeled mRNA, and the identified cells are selectively separated. In the present invention, there are no limitations to the method to detect the labeled mRNA. All of the live cell groups of which markers have been introduced do not always express the objective mRNA. In addition, when the marker has been introduced excessively in amount into the cells expressing the mRNA, the marker which does not bind to the mRNA will remain in the cell. Therefore, it is necessary to detect the labeled mRNA, i.e., markers bound to the mRNA, specifically among the unbound and free markers.

[0048]

As a method for easily and highly sensitively detecting labeled

mRNA in the presence of markers unbound to the mRNA, the first probe labeled with an energy donor dye and the second probe labeled with an energy acceptor dyes are preferably used together. These probes are introduced into the live cells to keep the live cells under the conditions where the probes and the mRNA can hybridize, the excitation light of the energy donor fluorescent dye of the first probe is irradiated to the live cell group, whereby the fluorescence from the energy acceptor fluorescent dye of the second probe is observed based on FRET, and finally the fluorescent labeled mRNA is detected specifically.

[0049]

Although irradiation of excitation light excites the energy donor dye of the first probe whether the probe hybridizes to the objective mRNA or not, only when the both of the first and second probes hybridize adjacently to the same mRNA, FRET occurs, resulting in emission of fluorescence from the energy acceptor fluorescent dye. That is, the FRET-fluorescence from the acceptor fluorescent dye indicates that the first probe and the second probe are adjacent on the objective mRNA, showing that the objective genes are expressed in the cells.

[0050]

The live cells which have expressed specific genes detected in this way are selectively separated, but there are no limitations for this separating method. In the present invention, when fluorescence-labeled probes are used, it is preferable to use a cell sorter (Fluorescence Activated Cell Sorter, FACS) to detect live cells, which express specific genes, and then to selectively separate them.

[0051]

In general, an apparatus consisting of a flow cytometer and a cell dispenser is called a cell sorter. Individual cells, which have been stained with fluorescence-labeled probes, are exposed to laser beam on the way of a flow path, resulting in the light scattering and fluorescence from the cell. The intensity of light scattering (forward scattering light or side scattering light) and fluorescence are measured for each cell and the results for a number of cells are displayed, for example, as a frequency distribution diagram (dot plot). Then, the cells emitting fluorescence with the desired extent are collected by gating. The method as mentioned above is called flow cytometry.

[0052]

In the present invention, when live cells which have expressed a specific gene are selectively separated with the cell sorter using fluorescent labeled probes which can cause FRET, the following method can be applied. In the cell sorter, laser exciting energy donor is irradiated to each cell, and fluorescence intensities of energy donor fluorescent dyes (for example, Bodipy 493/503) as well as on relative fluorescence intensities of energy acceptor dyes (for example, Cy5) are obtained. Each cell is represented as a dot according to the intensity of the donor and acceptor fluorescence, in a diagram where the horizontal and vertical axis represents intensity of donor and acceptor fluorescence, respectively. Then, in the dot-plotted diagram, the cells emitting relatively high fluorescence intensity on the vertical axis are selected and these cells are designated as a region in the diagram, R2. Next, obtaining the intensity of forward scattering light (FSC), and side scattering light (SSC) representing the size of live cells to be measured, and the complexities of cellular

intrastructures, respectively, each cell is represented as a dot in a diagram according to the value of FSC and SSC, on the horizontal and vertical axis, respectively. In the dot-plotted diagram, a region representing the cells emitting FSC as well as SSC with the desirable extent, is selected and designated as R1. After a cell sorter is set so that only live cells suitable for both conditions of R1 and R2 are collected, the cells expressing specific mRNA are selectively separated.

[0053]

By using the methods as described, various types of live cells as well as various type of mRNA are objectives of selective separation. TH1 and TH2 derived from helper T cells which have been activated by recognizing antigens as foreign do not have any crucial cell surface antigens (markers) to be distinguished from each other. Further, cytokines produced by TH1 and TH2, IL-2 and IL-4, respectively, do not remain in the cell or near the cell membrane and are liberated to the extracellular fluid. Thus, TH1 or TH2 is an ideal objective to which the selective separation of the present invention is applied. That is, the selective separation method according to the present invention is preferably used for live cell groups containing live cells having mRNA encoding cytokines.

[0054]

Especially, it is preferable that live cell groups containing live cells having mRNA encoding interleukin-2 (IL-2) are applied to this method of the present invention. To separate live cells expressing IL-2 mRNA, the first probe labeled with the energy donor fluorescent dye having a base sequence set forth in SEQ ID NO: 9 in the Sequence Listing and the

second probe labeled with the energy acceptor fluorescent dye having a base sequence set forth in SEQ ID NO: 10 in the Sequence Listing are used, and FRET generated by these probes is utilized as an index for selective separation.

[0055]

The base sequence set forth in SEQ ID NO: 9 in the Sequence Listing is complementary to the base sequence, 342-356 in the base sequence of mRNA encoding IL-2, while the base sequences set forth in SEQ ID NO: 10 in the Sequence Listing is complementary to the base sequence, 357-371 in the base sequence of mRNA encoding IL-2. When the first probe labeled with the energy donor fluorescent dye are made adjacent to the second probe labeled with the energy acceptor fluorescent dye at the positions indicated above of the same mRNA molecule, the detection of the FRET-fluorescence can be carried out with high sensitivity.

[0056]

Furthermore, it is preferable that live cell groups containing live cells having mRNA encoding interleukin-4 (IL-4) are are applied to this method of the present invention. To separate live cells expressing IL-4 mRNA, the first probe labeled with the energy donor fluorescent dye having a base sequence set forth in SEQ ID NO: 17 in the Sequence Listing and the second probe labeled with the energy acceptor fluorescent dye having a base sequence set forth in SEQ ID NO: 18 in the Sequence Listing are used, and FRET generated by these probes is utilized as an index for separation.

[0057]

The base sequence set forth in SEQ ID NO: 17 in the Sequence

Listing is complementary to the base sequence, 265-279 in the base sequence of mRNA encoding IL-4, while the base sequences set forth in SEQ ID NO: 18 in the Sequence Listing is complementary to the base sequence, 280-294 in the base sequence of mRNA encoding IL-4. When the first probe labeled with the energy donor fluorescent dye are made adjacent to the second probe labeled with the energy acceptor fluorescent dye at the positions indicated above of the same mRNA molecule, the detection of the FRET-fluorescence can be carried out with high sensitivity.

[0058]

[Examples]

The present invention will be explained in greater detail by way of preferred examples hereinbelow. However, the present invention should not be limited to these examples.

[0059]

(1) Preparation of fluorescent labeled markers of oligonucleotides complementary to IL-2 mRNA and IL-4 mRNAP

Five sites, 30 bases each, were selected on the base sequences of IL-2 or IL-4 mRNA. Each 30 bases-site was divided into two halves (15 bases-site). An oligonucleotide complementary to each 15 bases-site was designed as a DNA probe for each site and labeled with a fluorescent dye, Bodipy493/503, Cy5, or XRITC.

[0060]

The DNA probe was synthesized using a DNA/RNA synthesizer (Perkin Elmer: Model 394 or Perceptive: Model 18909), by β-cyanoethylamidethod. The entire base sequence of IL-2 mRNA and base sequences of the oligo DNA probes are shown in Fig. 1, and the entire

base sequence of IL-4 mRNA and base sequences of the oligo DNA probes are shown in Fig. 2. The base sequences of the designed 10 types of oligo DNA probes for IL-2 mRNA (SEQ ID NOs: 1-10) and the base numbers (hybridized positions) on IL-2 mRNA to which the oligo DNA probes hybridize are shown in Table 1. The base sequences of 10 types of oligo DNA probes for IL-4 mRNA (SEQ ID NOs: 11-20) and the base numbers (hybridized positions) on IL-4 mRNA to which the oligo DNA probes hybridize are shown in Table 2.

[0061]

Table 1

SEQ ID NO:	Base Sequence	Hybridized Position*
SEQ ID NO: 1	5'-GTAAAACTTAAATGT-3'	228-242
SEQ ID NO: 2	5'-GGCCTTCTTGGGCAT-3'	243-257
SEQ ID NO: 3	5'-TTTGGGATTCTTGTA-3'	198-212
SEQ ID NO: 4	5'-GAGCATCCTGGTGAG-3'	213-227
SEQ ID NO: 5	5'-GCAAGACTTAGTGCA-3'	77-91
SEQ ID NO: 6	5'-CTGTTTGTGACAAGT-3'	92-106
SEQ ID NO: 7	5'-GGTTTGAGTTCTTCT-3'	287-301
SEQ ID NO: 8	5'-AGCACTTCCTCCAGA-3'	302-316
SEQ ID NO: 9	5'-CCTGGGTCTTAAGTG-3'	342-356
SEQ ID NO: 10	5'-ATTGCTGATTAAGTC-3'	357-371

*:Base number of IL-2 mRNA to which each probe hybridizes.

[0062]

Table 2

SEQ ID NO:	Base Sequence	Hybridized Position*
SEQ ID NO: 11	5'-CAGTTGGGAGGTGAG-3'	70-84
SEQ ID NO: 12	5'-GAACAGAGGGGGAAG-3'	85-99
SEQ ID NO: 13	5'-CGTGGACAAAGTTGC-3'	119-133
SEQ ID NO: 14	5'-TATCGCACTTGTGTC-3'	134-148
SEQ ID NO: 15	5'-CTGTGAGGCTGTTCA-3'	176-190
SEQ ID NO: 16	5'-ACAGAGTCTTCTGCT-3'	191-205
SEQ ID NO: 17	5'-AGCCCTGCAGAAGGT-3'	265-279
SEQ ID NO: 18	5'-CCGGAGCACAGTCGC-3'	280-294
SEQ ID NO: 19	5'-CCGTTTCAGGAATCG-3'	376-390
SEQ ID NO: 20	5'-GAGGTTCCTGTCGAG-3'	391-405

*:Base number of IL-4 mRNA to which each probe hybridizes.

[0063]

In (4A) described below, oligo DNAs corresponding SEQ ID NOs: 1-10 were labeled with Bodipy493/503 at the 5' end, and were used as probes. In (8), unlabeled oligo DNAs corresponding SEQ ID NOs: 1-10 were used as probes. In (9), oligo DNAs corresponding SEQ ID NOs: 1, 3, 5, 7, or 9 were labeled with Bodipy493/503 at the 5' end, and oligo DNAs corresponding SEQ ID NOs: 2, 4, 6, 8, or 10 were labeled with XRITC at the linkage between the 4th nucleotide and the 5th from the 3' end and were used as probes. In (11), oligo DNAs corresponding SEQ ID NOs: 1, 3, 5, 7, or 9 were labeled with Bodipy493/503 at the 5' end, and oligo DNAs corresponding SEQ ID NOs: 2, 4, 6, 8, or 10 were labeled with Cy5

at the linkage between the 4th nucleotide and the 5th from the 3' end and were used as probes. The labeling of oligo DNAs with Bodipy493/503, XRITC, or Cy5 were performed as described in (a)-(c).

[0064]

In the present invention, the oligo DNA probes, which are labeled with energy donor fluorescent dyes, are sometimes abbreviated as donor probes, while oligo DNA probes, which are labeled with energy acceptor fluorescent dyes are sometimes abbreviated as acceptor probes. The probe, corresponding SEQ ID NO: 1 was labeled with an energy donor fluorescent dye, is complementary to the sequence, 228-242 of IL-2 mRNA. Thus, the probe may sometimes be referred to as IL-2 228-242(D) (D means a donor). The probe, corresponding SEQ ID NO: 2 was labeled with an energy acceptor fluorescent dye, is complementary to the sequence, 243-257 of IL-2 mRNA. Thus, the probe may be sometimes referred to as IL-2 243-257(A) (A means an acceptor). In addition, when the probe is not labeled with a fluorescent dye, it is simply referred to as IL-2 228-242. Thus, the probes used in (9) and (11) may be represented by the names of probes shown in Table 3 hereunder.

[0065]

Table 3

SEQ ID NO:	Base sequence	Name of Probes
SEQ ID NO: 1	5'-GTAAAACTTAAATGT-3'	IL-2 228-242(D)
SEQ ID NO: 2	5'-GGCCTTCTTGGGCAT-3'	IL-2 243-257(A)
SEQ ID NO: 3	5'-TTTGGGATTCTTGTA-3'	IL-2 198-212(D)
SEQ ID NO: 4	5'-GAGCATCCTGGTGAG-3'	IL-2 213-227(A)
SEQ ID NO: 5	5'-GCAAGACTTAGTGCA-3'	IL-2 77-91(D)
SEQ ID NO: 6	5'-CTGTTTGTGACAAGT-3'	IL-2 92-106(A)

SEQ ID NO: 7	5'-GGTTTGAGTTCTTCT-3'	IL-2 287-301(D)
SEQ ID NO: 8	5'-AGCACTTCCTCCAGA-3'	IL-2 302-316(A)
SEQ ID NO: 9	5'-CCTGGGTCTTAAGTG-3'	IL-2 342-356(D)
SEQ ID NO: 10	5'-ATTGCTGATTAAGTC-3'	IL-2 357-371(A)

[0066]

IL-4 probes are sometimes represented in the same manner as above. For example, the probes used in (3B) as described below may be represented by the names of probes shown in Table 4 hereunder.

[0067]

Table 4

SEQ ID NO:	Base Sequence	Name of Probes
SEQ ID NO: 11	5'-CAGTTGGGAGGTGAG-3'	IL-4 70-84(D)
SEQ ID NO: 12	5'-GAACAGAGGGGGAAG-3'	IL-4 85-99(A)
SEQ ID NO: 13	5'-CGTGGACAAAGTTGC-3'	IL-4 119-133(D)
SEQ ID NO: 14	5'-TATCGCACTTGTGTC-3'	IL-4 134-148(A)
SEQ ID NO: 15	5'-CTGTGAGGCTGTTCA-3'	IL-4 176-190(D)
SEQ ID NO: 16	5'-ACAGAGTCTTCTGCT-3'	IL-4 191-205(A)
SEQ ID NO: 17	5'-AGCCCTGCAGAAGGT-3'	IL-4 265-279(D)
SEQ ID NO: 18	5'-CCGGAGCACAGTCGC-3'	IL-4 280-294(A)
SEQ ID NO: 19	5'-CCGTTTCAGGAATCG-3'	IL-4 376-390(D)
SEQ ID NO: 20	5'-GAGGTTCCTGTCGAG-3'	IL-4 391-405(A)

[0068]

(a) Preparation of donor probes (Bodipy 493/503-labled)

2.5 mg of NHSS (N-Hydroxysulfosuccinimide sodium salt) in 30 μ l of sterilized water, 5 mg of EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] in 50 μ l of sterilized water, and 1 mg

of Bodipy493/503 propionic acid dissolved in 50 μ l of DMF were mixed and reacted with at room temperature for 30 minutes.

[0069]

An oligo DNA with the base sequence described above (lyophilized product), which a hexylamino group was introduced to the 5' end using 6-(trifluoroacetylamino)hexyl-(2-cyanoethyl)-(N,N-di-isopropyl)-phosphoroamidite, a 5' end aminating agent, was dissolved in 200 μ l of 0.5 M of Na_2HCO_3/NaH_2CO_3 buffer solution (pH 9.3).

[0070]

These were mixed and reacted overnight in the dark. After the reacted solution was gel filtrated to remove unreacted dyes, the reaction solution was subjected to reversed phase high performance liquid chromatography (HPLC) with CAPCELL PACK18 (Shiseido Inc., Column size: 6 mm in inner diameter x 250 mm in length), and the fractions with absorption at 260nm and 493nm were recovered and lyophilized. HPLC was performed under the following condition, flow rate; 1 ml/minute, temperature; 40°C, the mobile phase was the mixture of solution A (5% CH_3CN containing 5 mM of TEAA) and solution B (40% CH_3CN) and the concentration gradient of CH_3CN was generated by increasing the concentration of solution B from 30% to 80 in 20 minutes.

[0071]

(b) Preparation of acceptor probes (Cy5-labeled)

Cy5 dye in one tube (Amersham, Fluorolink Cat.No.PA25001) was dissolved in 100 μ l of sterilized water. An oligo DNA with the base sequence described above (lyophilized product) which a hexylamino

group was introduced into the linkage between the 4th nucleotide and the 5th from the 3' end using a Uni-Link AminoModifier (Clontech Inc.), was dissolved in 200 μl of Na_2HCO_3/NaH_2CO_3 buffer solution (0.5 M, pH 9.3). These were mixed in the dark, and reacted overnight.

[0072]

After the reacted solution was gel filtrated to remove unreacted dyes, the reaction solution was subjected to reversed phase high performance liquid chromatography (HPLC) with CAPCELL PACK18 (Shiseido Inc., Column size: 6 mm in inner diameter x 250 mm in length), and the fractions with absorption at 260nm were recovered. HPLC was performed under the following condition, flow rate; 1 ml/minute, temperature; 40°C, the mobile phase was the mixture of solution A (5% CH_3CN containing 5 mM of TEAA) and solution B (40% CH_3CN) and the concentration gradient of CH_3CN was generated by increasing the concentration of solution B from 15% to 60 in 20 minutes. When the absorption spectra of the recovered fractions was measured in the range of 220-700nm, the maximum absorption was observed between 650-700nm, indicating a typical property of Cy5. And then the fractions were lyophilized.

[0073]

(c) Preparation of acceptor probes (XRITC-labeld)

One hundred microliters of XRITC dye solution (Solvent: 100% DMSO, Perkin Elmer, ROX-NHS) was reacted with an oligo DNA having the base sequence described above where a hexylamino group was introduced as described in (B). The reaction product was applied to reversed phase high performance liquid chromatography; a fraction with

an absorption band at 260nm was recovered; the absorption spectrum was measured in the range between 220-650 nm; after the maximum absorption of XRITC was observed in the range between 550-600 nm, the recovered fraction was lyophilized.

[0074]

(2A) In vitro synthesis of human IL-2 RNA

In order to obtain human IL-2 RNA having a base sequence equivalent to human IL-2 mRNA, an IL-2 cDNA fragment was cleaved out with restriction enzyme pst I from pTCGF-II (ATCC#39673), a plasmid containing a human IL-2 cDNA and was linked to the pst I site of pBluescript KS(+) a plasmid vector for RNA synthesis using Ligation kit version 2 (Takara) so that the cDNA would be located in the downstream of T3 promoter. The obtained recombinant plasmid was introduced into competent cells of *E. coli* JM109 strain (Takara Co.), and the transformants of the *E. coli* obtained were cultured, 46.2 μ g of the plasmid DNA was extracted and purified from 100 ml of the bacterial culture using a Plasmid Midi Kit (QIAGEN).

[0075]

The recombinant plasmid was linearized by restriction enzyme Sma I digestion to prepare the template for the synthesis of human IL-2 RNA. The enzyme proteins in the plasmid solution was degraded with proteinase K and denatured/removed with phenol/chloroform. The RNA synthesis was performed using 0.66 μ g of the purified template with the base composition, A (adenine) : C (cytosine) : G (guanine) : U (uracil), 35 : 18 : 14 : 32%. A, C, G and U were added to the template at the final concentrations of 105, 54, 42 and 96 mM, respectively together with T3

RNA polymerase according to the aid of an in vitro transcription kit (Megascript T3 Kits, Ambion). Polymerase reaction was carried out at 37 °C for 6 hours to synthesize human IL-2 RNA. After the reaction was over, the RNA was purified as follows. The template DNA was decomposed with DNase I (Megascript T3 Kits, Ambion Inc.), the enzyme proteins in the transcription reaction solution was denatured/removed with phenol/chloroform. To the obtained RNA solution was added an equal volume of isopropanol, and human IL-2 RNA was recovered as a precipitate by centrifugation (14 krpm, for 7 minutes), while the respective nucleotides which were unreacted enzyme substrates were removed. The human IL-2 RNA precipitate (139 μg) rinsed once with 70% ethanol was dissolved in RNase-free water (Megascript T3 Kits, Ambion) so that 5 μg/μl of human IL-2 RNA solution was prepared to use for the subsequent hybridization experiments.

[0076]

(2B) In vitro synthesis of human IL-4 RNA

Next, in order to obtain human IL-4 RNA a having base sequence equivalent to human IL-4 mRNA, human IL-4 cDNA fragment was cleaved out with restriction enzymes BamHI and Xho I from pcD-hIL-4 (ATCC#57593), a plasmid DNA containing human IL-4 cDNA. The cDNA fragment was linked to the BamHI and Xho I sites of a pBluescript KS(+), a plasmid vector for RNA synthesis using Ligation kit version 2 (Takara) so that the cDNA would be located in the downstream of T3 promoter. The obtained recombinant plasmid was introduced into competent cells of *E. coli* JM109 strain (Takara Co.); the transformants of the *E. coli* obtained

were cultured; and 152 μg of plasmid DNA was extracted and purified from 100 ml of the bacterial culture using a Plasmid Midi Kit (QIAGEN).

[0077]

The recombinant plasmid was linearized by digestion with restriction enzyme Sma I to prepare the template for the synthesis of human IL-4 RNA. The enzyme proteins in the plasmid solution was degraded with proteinase K and denatured/removed with phenol/chloroform. The RNA synthesis was performed using 0.46 μg of the purified template with the base composition, A : C : G : U, 29 : 24 : 21 : 26%. A, C, G and U were added to the template at the final concentrations of 87, 72, 63 and 78 mM, respectively together with T7 RNA polymerase according to the aid of an in vitro transcription kit (Megascript T7 Kits, Ambion). Polymerase reaction was carried out at 37 °C for 6 hours to synthesize human IL-4 RNA. After the reaction was over, the RNA was purified as follows.

[0078]

The template DNA was decomposed with DNase I (Megascript T7 Kits, Ambion Inc.), the enzyme proteins in the transcription reaction solution was denatured/removed with phenol/chloroform. To the obtained RNA solution was added an equal volume of isopropanol, and human IL-4 RNA was recovered as a precipitate by centrifugation (14 krpm, for 7 minutes), while the free nucleotides, unreacted enzyme substrates, were removed. The human IL-4 RNA precipitate (139 μg) rinsed once with 70% ethanol was dissolved in RNase-free water (Megascript T7 Kits, Ambion) so that 5 μg/μl of human IL-4 RNA solution was prepared to use for the subsequent hybridization experiments.

[0079]

(3A) Changes in fluorescence spectra by hybridization of fluorescent labeled probes to human IL-2 RNA

In order to measure changes in fluorescence spectra due to fluorescence resonance energy transfer (FRET) caused by hybridization of donor probes and acceptor probes to adjacent sites on IL-2 RNA, a pair of 300 nM (final concentration) of Bodipy 493/503-labeld donor probes and XRITC-labeled acceptor probes, and human IL-2 RNA were mixed in 100 μ l of 1 x SSC solution (150 mM sodium chloride, 17 mM citric acid, pH 7.0), and allowed to stand at room temperature for 15 minutes, and then fluorescence spectra were measured. As combinations of donor probes and acceptor probes, IL-2 228-242(D) and IL-2 243-257(A), IL-2 198-212(D) and IL-2 213-227(A), IL-2 77-91(D) and IL-2 92-106(A), IL-2 287-301(D) and IL-2 302-316(A), and IL-2 342-356(D) and IL-2 357-371(A), were used. As a control, the fluorescence spectrum of 300 nM of the probe alone was also measured. The conditions for fluorescence spectrum measurement were as follows:

Fluorospectrophotometer: F4500 (Hitachi)

108 Excitation wavelength: 480nm

Fluorescence-measurement Wavelength: 500-750 nm

Temperature: room temperature

[0080]

For all the combinations examined, changes in fluorescence spectra were observed, i.e., the intensity of donor fluorescence decreased while acceptor fluorescence(580-650nm) increased with donor excitation due to the addition of human IL-2 RNA to the probe solution. See Figs. 3-7.

Figs. 3, 4, 5, 6 and 7 show fluorescence spectra when the combinations of IL-2 228-242 (D) and IL-2 243-257(A), IL-2 198-212(D) and IL-2 213-227(A), IL-2 77-91(D) and IL-2 92-106(A), IL-2 287-301(D) and IL-2 302-316(A), and IL-2 342-356(D) and IL-2 357-371(A) were used. As seen from the comparison among Figs. 2-6, the extent of changes in fluorescence spectra was different from each other among the combinations of probes, the most remarkable change occurred when the combination of IL-2 342-356(D) and IL-2 357-371(A) was used.

[0081]

(3B) Changes in fluorescence spectra by hybridization of fluorescent labeled probes to human IL-4 RNA

In order to measure changes in fluorescence spectra due to fluorescence resonance energy transfer (FRET) caused by hybridization of donor probes and acceptor probes to adjacent sites on IL-4 RNA, a pair of 300 nM (final concentration) of Bodipy 493/503-labed donor probes and Cy5-labeled acceptor probe, and human IL-4 RNA were mixed in 100 μl of 1 x SSC solution (150 mM sodium chloride, 17 mM citric acid, pH 7.0), and allowed to stand at room temperature for 15 minutes, and then fluorescence spectra were measured. As combinations of donor probes and acceptor probes, IL-4 70-84(D) and IL-4 85-99(A), IL-4 119-133(D) and IL-4 134-148(A), IL-4 176-190(D) and IL-4 191-205(A), IL-4 265-279(D) and IL-4 280-294(A), and IL-4 376-390(D) and IL-4 391-405(A), were used. As a control, the fluorescence spectrum of 300 nM of the probe described above alone was also measured. The conditions for measurement were as follows:

Fluorospectrophotometer: F4500 (Hitachi)

Excitation wavelength: 480nm

Fluorescence-measurement Wavelength: 500-750 nm

Temperature: room temperature

[0082]

For all the combinations examined, changes in fluorescence spectra were observed, i.e., the intensity of donor fluorescence decreased while acceptor fluorescence(650-700nm) increased with donor excitation due to the addition of human IL-4 RNA to the probe solution. See Figs. 8-12. Figs. 8, 9, 10, 11 and 12 show fluorescence spectra when the combinations of IL-4 70-84 (D) and IL-4 85-99(A), IL-4 119-133(D) and IL-4 134-148(A), IL-4 176-190(D) and IL-4 191-205(A), IL-4 265-279(D) and IL-4 280-294(A), and IL-4 376-390(D) and IL-4 391-405(A) were used. As seen from the comparison among Figs. 8-12, the extent of changes in fluorescence spectra was different from each other among the combinations of probes, the most remarkable change occurred when the combination of IL-4 265-279(D) and IL-4 280-294(A) was used. The results of the measurements are shown as the relative fluorescence intensity of Cy5 to Bodipy493-503 by excitation of Bodipy493-503 (Cy5 fluorescence intensity/Bodipy493-503 fluorescence intensity) in Table 5.

[0083]

Table 5

Probe Combination	Ratio of Fluorescence Intensities
IL-4 70-84(D) and IL-4 85-99(A)	1.3%
IL-4 119-133(D) and IL-4 134-148(A)	2.5%
IL-4 176-190(D) and IL-4 191-205(A)	22.4%
IL-4 265-279(D) and IL-4 280-294(A)	14.5%

IL-4 376-390(D) and IL-4 391-405(A)	1.5%

[0084]

(4A) Measurement of hybridization efficiency of probes to human IL-2 RNA by HPLC

Each donor probe (3 pmol) wherein an oligo DNA corresponding SEQ ID NO: 1-10 was labeled with Bodipy493/503 was mixed with an equimolar amount of human IL-2 RNA synthesized in (2A) in 10 μ l of 1 X SSC solution (150 mM sodium chloride, 17 mM sodium citrate, pH 7.0), and the mixture was allowed to stand at room temperature for 15 minutes. Subsequently, hybrids consisting of human IL-2 RNA and probes were separated from free probes by high performance liquid chromatography (HPLC) using differences in retention time under the following conditions, i.e., retention time is about 4-5 minutes and 7.5 minutes for free probes and hybrids , respectively.

Column: TSKgel DRAE-NPR (Toso Inc., 4.6 mm in inner Diameter X 35 mm in total length)

Flow Rate: 1 ml/ minute

Temperature: 25°C

Mobile phase: Solution A: 20 mM Tris-HCl (pH 9.0),

Solution B: 0.5 M NaCl, 20 mM Tris-HCl (pH 9.0)

[0085]

HPLC was performed in the concentration gradient manner. The mobile phase was the mixture of solution A and solution B and the concentration gradient of NaCl was generated by increasing the concentration of solution B from 25% to 100% so that the concentration of

NaCl changed from 0.125 M to 0.5M in 10 minutes. Absorbance at 260nm for nucleic acids and fluorescence intensity at 515nm with the excitation at 475nm for Bodipy493/503 were monitored simultaneously on eluted fractions. The fractions with the absorbance at 260nm as well as the fluorescence at 515nm were regarded as the ones of hybrids. The relative fluorescence intensity of the hybrid fractions to all the fractions in the fluorescence chromatogram was estimated and used as an index for the efficiency of hybridization.

[0086]

Fig. 13 shows a HPLC chromatogram when IL-2 342-356 (D) was mixed with IL-2 RNA. Fig. 14 shows a HPLC chromatogram when IL-2 357-371(D) was mixed with IL-2 RNA. Ratios of the peak areas of hybrids to all the peak areas in the fluorescence chromatogram, estimated for each probe, were summarized and shown in Table 6.

[0087]

Table 6

Name of Probe	Base Sequence	Ratio of Hybrid(%)

IL-2 228-242(D)	5'-GTAAAACTTAAATGT-3'	0.1
IL-2 243-257(D)	5'-GGCCTTCTTGGGCAT-3'	17.5
IL-2 198-212(D)	5'-TTTGGGATTCTTGTA-3'	15.7
IL-2 213-227(D)	5'-GAGCATCCTGGTGAG-3'	25.2
IL-2 77-91(D)	5'-GCAAGACTTAGTGCA-3'	0.5
IL-2 92-106(D)	5'-CTGTTTGTGACAAGT-3'	18.3
IL-2 287-301(D)	5'-GGTTTGAGTTCTTCT-3'	13.3
IL-2 302-316(D)	5'-AGCACTTCCTCCAGA-3'	6.2
IL-2 342-356(D)	5'-CCTGGGTCTTAAGTG-3'	22.8
IL-2 357-371(D)	5'-ATTGCTGATTAAGTC-3'	27.3

[0088]

From the results shown in Table 6, it was found that IL-2 213-227(D), IL-2 342-356(D), and IL-2 357-371(D) were hybridized to the target RNA with relatively high efficiency. In the results of (3A), the combination of IL-2 342-356(D) and IL-2 357-371(A) caused the largest changes in fluorescence spectra as a pair of a donor probe and an acceptor probe when the probes were mixed with IL-2 RNA. Therefore, the results of (4A) are well consistent with those of (3A).

[0089]

(4B) Measurement of hybridization efficiency of probes to human IL-4 RNA by HPLC

Each donor probe (3 pmol) wherein an oligo DNA corresponding SEQ ID NO: 11-20 was labeled with Bodipy493/503 was mixed with an equimolar amount of human IL-4 RNA, synthesized in (2B) in 10 μl of 1 X SSC solution (150 mM sodium chloride, 17 mM sodium citrate, pH 7.0) and the mixture was allowed to stand at room temperature for 15 minutes. Then, hybrids consisting of human IL-4 RNA and probes were separated from

free probes by high performance liquid chromatography (HPLC) using differences in retention time under the following conditions: retention time is about 4-5 minutes for free probe and about 7.5 minutes for hybrid in the HPLC condition below. The conditions for separation and the method for determining hybridization efficiency are the same as those described in (4A).

[0090]

Figs. 15, 16, 17 and 18 show HPLC chromatograms when IL-4 119-133 (D), IL-4 134-148(D), IL-4 265-279(D), and IL-4 280-294(D) were mixed with IL-4 RNA, respectively. Ratios based on the peak areas of hybrids to all the peak areas in the fluorescence chromatogram, estimated for each probe, were summarized and shown in Table 7.

[0091]

Table 7

Hybridized Position	Base Sequence	Ratio of Hybrid (%)
IL-4 70-84(D)	5'-CAGTTGGGAGGTGAG-3'	68.6
IL-4 85-99(D)	5'-GAACAGAGGGGGAAG-3'	53.5
IL-4 119-133(D)	5'-CGTGGACAAAGTTGC-3'	4.2
IL-4 134-148(D)	5'-TATCGCACTTGTGTC-3'	22.6
IL-4 176-190(D)	5'-CTGTGAGGCTGTTCA-3'	23.7
IL-4 191-205(D)	5'-ACAGAGTCTTCTGCT-3'	1.5
IL-4 265-279(D)	5'-AGCCCTGCAGAAGGT-3'	15.6
IL-4 280-294(D)	5'-CCGGAGCACAGTCGC-3'	46.6
IL-4 376-390(D)	5'-CCGTTTCAGGAATCG-3'	23.1
IL-4 391-405(D)	5'-GAGGTTCCTGTCGAG-3'	4.0

[0092]

From the results shown in Table 7, it was found that IL-4 265-279(D) and IL-4 280-294(D) were hybridized to the target RNA with relatively high efficiency. In the results of (3B), the combination of IL-4 265-279(D) and IL-4 280-294(A) caused the largest changes in fluorescence spectra as a pair of a donor probe and an acceptor probe when the probes were mixed with IL-4 RNA. Therefore, the results of (4B) are well consistent with those of (3B).

[0093]

(5) Induction of IL-2 gene expression in human T-cell leukemia strain cells Jurkat E6-1

To Jurkat E6-1 cells with a cell density of $1X10^6$/ml was added 0.5 mg/ml (final concentration) of anti-CD3 antibody (Immunotech Inc.), 0.5 mg/ml anti-CD28 antibody (Immunotech Inc.), and 10 nM PMA (Sigma Inc.), and they were cultured for 3 days (24 hours) at 37 °C in the presence of 5% CO_2.

[0094]

(6) Measurement of the production amount of IL-2 protein molecules

If a large amount of IL-2 molecules is produced and liberated into culture supernatant in response to the induction of IL-2 gene expression in (5), it is plausible that IL-2 mRNA is actively synthesized in the cells. Thus, in order to confirm IL-2 gene expression, culture supernatant of Jurkat E6-1 cells treated with IL-2 expression-inducing agents (in some cases, hereinafter called IL-2 expression-induced cells) which had been treated as described in (5) was collected; the amount of IL-2 (pg/ml/10^7 cells) in the supernatant was determined by the ELISA sandwich method (which will be described below) using Human interleukin-2 measurement kit

(Japan Immunoresearch Laboratories Co.,Ltd.) and the amounts of IL-2 in the supernatant of the treated cells were compared with those for untreated cells (in some cases, hereinafter called IL-2 expression-uninduced cells).

[0095]

Wells in a 96-well plate (antibody plate) on which anti-human IL-2 monoclonal antibodies were immobilized were washed with washing solution twice, and 150 μl of buffer solution was added to each well to be used. To each well was added 50 μl of the supernatant of culture medium or purified IL-2 (0 - 1,600 pg/ml, standard human IL-2 protein in Human IL-2 measurement kit), they were incubated at 37 °C overnight. The reaction solution in each well was removed, and the well was washed with washing solution three times. The first antibody (anti-human IL-2 rabbit serum) solution was added at 100 μl/well, and incubated at room temperature for 2 hours. The antibody solution in each well was removed, followed by washing with the washing solution three times.

[0096]

The second antibody (peroxidase labeled anti-rabbit IgG antibodies) solution was added at 100 μl/well, and incubated at room temperature for 2 hours. The antibody solution in each well was removed. After the well was washed with washing solution three times, and fully dried. A peroxidase substrate solution, o-phenylenediamine dissolved in 0.015% hydrogen peroxide was added at 100 μl/well, and was allowed to react at room temperature for 10-20 minutes. 100 μl of 1 N H_2SO_4 was added to each well to stop the reaction. Absorbance at 492 nm in each well was measured using a microplate reader. IL-2 in the culture

supernatant was quantified based on the calibration curve created from the values of absorbance of standard IL-2.

[0097]

6157 $\pm$ 168 (pg/ml/10^7 cells) of IL-2 were detected in the culture medium of IL-2 expression-induced cells, while the amount of IL-2 was less than the detectable range for IL-2 expression-uninduced cells (<0.1pg/ml/10^7 cells).

[0098]

(7) Measurement of amounts of IL-2 gene expression

For experimental materials, this experiment required purified IL-2 RNA as a reference sample, the total RNA extracted from Jurkat B6-1 cells as a measurement sample, and ribonucleic acid probe of IL-2 (RNA probe for IL-2) labeled with digoxigenin for the detection of IL-2 RNA or IL-2 mRNA. They were obtained using the methods in the following (a)-(c).

[0099]

(a) Standard IL-2 RNA

Human IL-2 RNA (1 μ g/ μ l) synthesized in (2) was diluted by 10^4, 10^5, 10^6, and 10^7 times with 1xdilution buffer (RNase-free sterile distilled water: 20 x SSC: formamide = 5:3:1). The diluted RNA solutions were heated at 68 °C for 10 minutes, then quenched on ice, and then used for blotting.

[0100]

(b) Total RNA of Jurkat E6-1 cells

Total RNA of Jurkat E6-1 cell was extracted using an RNeasy kit (QIAGEN Inc.). Under the conditions shown in (5), cells treated with the IL-2 expression-inducing agent for 0, 24, 48, 72, and 96 hours (0.8-

$1.2x10^7$cells) were recovered as precipitates by centrifugation at 1,500 rpm for 5 minutes. The cells were suspended in 1,000 μl of homogenization buffer containing 10 μl of β-mercaptethanol and were denatured sufficiently by repeating a manipulation of suction/emission with a syringe in 18-gauges. To the homogenate was added 1,000 μl of 70% ethanol, then it was applied to a column for RNA absorption, centrifuged at 4,000 x g for 5 minutes, and then the column was washed once by centrifugation after the addition of washing buffer.

[0101]

RNase-free sterile distilled water was added to the column to elute the absorbed RNA. To the eluted RNA solution was added 0.1 times volume of 4 M sodium acetate and an equal volume of isopropanol. Then RNA was recovered as a precipitate by centrifugation at 15,000 x g for 15 minutes and. After the RNA was dissolved in the RNase-free sterile distilled water, it was diluted with an equal volume of 2 X dilution buffer (RNase-free sterile distilled water : 20 x SSC : formamide = 1 : 6 : 2), heated at 68 °C for 10 minutes and then quenched quickly.

[0102]

(c) Digoxigenin (DIG)-Labeled RNA probe for IL-2 RNA

DIG-labeled RNA probe for IL-2 RNA was synthesized using a DIG RNA Labeling kit (Boehringer Mannheim Inc.). 10 μg of human IL-2 cDNA recombinant plasmid DNA (pTCGF#2), linearized by EcoRI digestion, was purified by ethanol precipitation. After removing the enzyme protein denatured with phenol/chloroform, the purified DNA was used as a template for RNA probe synthesis. The template DNA (5 μg) and 1.8 mM ATP, 0.9 mM CTP, 0.7 mM GTP, 1.1 mM UTP, and 0.58 mM UTP (DIG-

labeled) were mixed in the presence of T7 RNA polymerase. The mixture was incubated at 37 °C for 2 hours. Then, DNase I solution was added and reacted for 10 minutes to degrade the template DNA. To the reaction solution were added 0.1 times volume of 5 M sodium acetate and an equal volume of isopropanol, and the synthesized RNA was recovered by centrifugation at 15,000xg for 15minuts. The RNA was then dissolved in RNase-free sterile distilled water.

[0103]

(d) Measurement of amounts of IL-2 gene expression

Using the materials obtained from (a)-(c), the amount of IL-2 gene expression was measured. The cellular total RNA solution and standard IL-2 RNA solution were dotted to a nylon membrane, and washed with 5 x SSC twice. Then, the RNA on the nylon membrane was fixed using a UV-Crosslinker (Biorad Inc.). The nylon membrane, prehybridization buffer (5XSSC, 5% SDS, 50 mM sodium phosphate (pH 7.0), 50% formamide, 2% Blocking Reagent (Boehringer Mannheim Inc.), and 1% N-lauryl sarcosinate were enclosed into a HybriBag (Iuchi Inc., Hot water resistant bag: L) and incubated at 68 °C for one hour.

[0104]

DIG-labeled probe for IL-2 RNA was diluted with a prehybridization buffer to give a final concentration of 100 ng/ml, boiled for 10 minutes and then quenched to prepare hybridization solution. Prehybridization solution in HybriBag was replaced with the hybridization solution, and hybridization was carried out at 68 °C overnight. The nylon membrane was washed with 2 X Washing solution (2 X SSC, 0.1% SDS) twice for 5 minutes each, and with 0.2 X Washing solution (0.2 X SSC, 0.1% SDS) at 68 °C for 15

minutes each. After washing the nylon membrane with Buffer I (100 mM maleic acid, 150 M NaCl (pH 7.5)) for one minute, the nylon membrane was incubated with Buffer II (Blocking Reagent (Boehringer Mannheim Inc.) was diluted to 1% with buffer I).

[0105]

The amounts of hybrid formed with DIG-labeled probes and IL-2 RNA, were estimated by chemiluminescence emitted from the hybrids using DIG Luminescent Detection kit (Boehringer Mannheim Inc.) as follows. The nylon membrane was treated with alkalinephosphatase-labeled anti-DIG antibodies (150 mU/ml in Buffer II solution), at room temperature for 1 hour. Then the nylon membrane was washed with Buffer I twice for 15 minutes each, and packed into a bag (LIFETECHNOLOGIES Inc., Photogene development folder) with 250 μ M of substrate solution which had been obtained by diluting CSPD (disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan]-4-yl) phenylphosphate) with Buffer III(0.1 M Tris, pH 9,7, 0,1 M NaCl, 0.05 $MgCl_2$). The number of photons emitted from the enzymatically degraded substrate was counted with ARGUS 50 (Hamamatsu Photonics). A calibration curve indicating the relation between the number of photon and the amount of standard IL-2 RNA was created. Based on the curve, the amount of IL-2 mRNA out of total cellular RNA in the cell was determined. From the amount of IL-2 mRNA (mol) obtained and the number of cells used for extraction of the total cellular RNA, the number of IL-2 mRNA molecules per single cell was determined (Fig. 19).

[0106]

As shown in Fig. 9, the numbers of IL-2 mRNA molecules in the

single cell where the cells were treated for inducing IL-2 expression for 0, 24, 48, 72, and 96 hours were <0.29, $(0.76 \pm 0.17) \times 10^4$, $(1.11 \pm 0.40) \times 10^4$, $(1.22 \pm 0.67) \times 10^4$, and $(1.20 \pm 0.28) \times 10^4$, respectively. The cells treated for 72 hours (3 days) were found to contain the maximum number of IL-2 mRNA. In addition, the contents of extracellular IL-2 (pg/ml/10^7 cells) of the cells treated for the above described periods were <0.32, $1,032 \pm 25$, $2,433 \pm 533$, $2,688 \pm 194$, and $2,531 \pm 283$, respectively. Therefore, it was suggested that more IL-2 molecules had been secreted from the cells with higher efficiency in IL-2 gene expression.

[0107]

(8) Intracellular hybridization between each probe and IL-2 mRNA in human T-cell leukemia strain Jurkat E6-1 cells induced the expression of IL-2 gene

For IL-2 expression-induced cells the IL-2 gene expression of which was induced by treatment with anti-CD3 antibodies, anti-CD28 antibodies, and PMA for 3 days as described in (5), detection of the hybridization between intracellular IL-2 mRNA and each IL-2 probe was performed by IST (In Situ Transcription) as follows.

[0108]

The IL-2 expression-induced and -uninduced Jurkat E6-1 cells (5×10^5 cells/ml) were washed with PBS(-) three times, and suspended with 1 ml of PBS(-), and the suspension was mounted on 12 mm of a cover glass (poly-L-lysine was coated on the bottom) to prepare a monolayer of cells. The cells were exposed to 0.5% Triton-X100 solution for 90 seconds at room temperature to permeabilize the cells. After the permeabilized cells were quickly washed with PBS(-), 10 μM (final concentration) of

probes corresponding to SEQ ID NO: 1-10 in Table 1 (unlabeled with dye), oligo dT (deoxythymidine oligonucleotide, unlabeled with dye) or oligo dA (deoxyadnine oligonucleotide, unlabeled with dye) were added, and incubated for one hour at room temperature. The cells were washed with PBS(-) quickly, and fixed with 4% parafromaldehyde solution at room temperature for 15 minutes.

[0109]

The cells were washed with 1 X SSC three times; 1 mM deoxyribonucleotide solution (Boehringer Mannheim Inc.) containing 0.35 mM of DIG (digoxigenin)-labeled dUTP and 1 u/ μ l reverse transcriptase (Toyobo, Inc.) were added to the cells; and they were incubated for 2 hours at 30 °C. The cells were washed with 1 X SSC three times, and treated with Blocking buffer [Blocking Reagent (Boehringer Mannheim Inc.) dissolved in maleic acid buffer solution so that its ratio would be equal to 1(w/v)%]. The cells were washed with maleic acid buffer solution three times. FITC (Fluorescein-isothiocyanate) labeled anti-DIG antibody (which was diluted with Blocking buffer up to 1 μ g/ml) was added to the cells and then the cells were incubated for 30 minutes at room temperature. The cover glass was washed with PBS(-) three times, observed under a fluorescence microscope, and the total fluorescence intensity in the visual field (relative value) was measured.

[0110]

Figs. 20 and 21 show the fluorescence micrographs obtained. Fig. 20 shows a fluorescence micrographs where the hybrids formed between all of the cellular mRNA and oligo dT were fluorescently detected in the fixed IL-2 expression-induced cells and -uninduced cells. Fig. 20 also

shows the control experiments in cases where oligo dA was added instead of oligo dT, or where both DIG-labeled dUTP and reverse transcriptase required for fluorescent labeled complex were not added. Fig. 21 shows fluorescence micrographs in which hybrids, formed between IL-2 mRNA and each probe (fluorescently unlabeled), were fluorescently labeled in the fixed IL-2 expression-induced and -uninduced. In Fig. 21, two fluorescence images for each probe are shown for IL-2 expression-induced and -uninduced cells. Fig. 22 shows the normalized intensity (%) of each probe out of the fluorescence intensity obtained from the addition of oligo dT to the cells, the relative fluorescent intensity per cell was worked out from the fluorescence intensities (mean value $\pm$ SE) divided by the number of cells in the corresponding phase contrast micrograph.

[0111]

IL-2 342-356 and IL-2 357-371 were shown to hybridize with intracellular IL-2 mRNA most efficiently. As these two probes are complementary to an adjacent site on IL-2 mRNA, if one of the two probes is labeled with an fluorescent energy donor dye and the other is labeled with an fluorescent energy acceptor dye, FRET fluorescence caused by intracellular hybridization could be detected specifically.

[0112]

<u>(9) Intracellular hybridization (ISH) of donor probes and acceptor probes with IL-2 mRNA in IL-2 expression-induced cells</u>

In the experimental results by IST in (8), the probes were introduced into almost all the cells uniformly in a visual field of a fluorescence microscope. The method for introducing probes in (8) was also used when fluorescent labeled probes of donors and acceptors were

introduced into cells. Hybridization between these probes and intracellular IL-2 mRNA was detected as FRET fluorescence. Various sets of probes were examined. Since this method modifies the IST method, it is called ISH (In Situ Hybridization).

[0113]

A solution of 2 μ M (final concentration) of a donor probe labeled with Bodipy 493/503 and an acceptor probe labeled with XRITC was added to IL-2 expression-induced cells as well as to IL-2 expression-uninduced cells, both of which were provided with material permeability in the cellular transmembranes as described in (8), and they were incubated for one hour at room temperature. As sets of donor probes and acceptor probes, IL-2 228-242(D) and IL-2 243-257(A), IL-2 198-212(D) and IL-2 213-227(A), IL-2 77-91(D) and IL-2 92-106(A), IL-2 287-301(D) and IL-2 302-316(A), and IL-2 342-356(D) and IL-2 357-371(A) were used. After the cells were washed with PBS(-), the cells were fixed with 4% paraformaldehyde solution for 15 minutes at room temperature. The cover glass was washed with PBS(-) three times before fluorescence microscopy. Three kinds of fluorescence images were obtained as follows. These are the fluorescence of A emitted from the cells when the excitation light of A (energy acceptor dye) was irradiated to the cells (in some cases, hereinafter called A/A image), the fluorescence of D emitted from the cells when the excitation light of D (energy donor dye) was irradiated to the cells (hereinafter, sometimes called D/D image), and the fluorescence of A emitted from the cells when the excitation light of D was irradiated to the cells (in some cases, hereinafter called D/A image). The D/A image represents fluorescence caused by FRET. The maximum intensity of

FRET fluorescence was obtained when IL-2 342-356(D) and was used as a donor probe and IL-2 357-371(A) as an acceptor probe. These fluorescence micrographs are shown in Fig. 23. In Fig. 23, two sets of D/A, A/A, and D/D images for each pair of probes are shown for IL-2 expression-induced and -uninduced cells. In this figure, the images in which probes were not introduced are also shown as a control experiment.

[0114]

The total fluorescence intensity in a D/A image, acceptor fluorescence of FRET with donor excitation, and a A/A image, acceptor fluorescence with acceptor excitation, were obtained. Then, the average fluorescence intensity value per cell in a D/A and A/A image was estimated as the total fluorescence intensity divided by the number of cells in a corresponding image. In order to evaluate the intracellular hybridization efficiency, the average D/A value per cell / the average A/A value per cell, representing the ratio of the hybridized acceptor probes to IL-2 mRNA to the total probes, were obtained. The value of (D/A)/(A/A) (%) for each pair of probes is shown in Fig. 24. From the results in (8) and (9), it is suggested that IL-2 342-356(D) and IL-2 357-371(A) hybridize to the target mRNA in the cells individually as well as adjacently.

[0115]

<u>(10) Intracellular hybridization when donor probes and acceptor probes were introduced into live IL-2 expression-induced cells</u>

Throughout the results of (3A)-(9), IL-2 342-356(D) and IL-2 357-371(A) were selected as probes to detect IL-2 mRNA in live cells. These fluorescently labeled probes were introduced into live cells expressing IL-2 genes; and the hybridization was specifically measured

based on the changes in FRET fluorescence.

[0116]

As described in (5), IL-2 expression-induced cells which were prepared by the treatment with anti-CD3 antibodies, anti-CD28 antibodies and PMA for three days, and -uninduced cells, were washed with ice-cold PBS(-) twice, and suspended in PBS(-) to 1×10^7 cells/ml. Then, 0.9 ml of the cell suspension was transferred into a cuvette for electroporation; and 5.4 nmol (final concentration; 6.0 μ M) of Bodipy493/503-labeled donor probe IL-2 342-356(D) and 5.3 nmol (final concentration; 5.86 μ M) of XRITC-labeled acceptor probe Il-2 357-371(A) were added; and the cells were pulsed at 250 V, 975 μ F. After the cell suspension was filtered through 70 μ m of Cell Strainer (Falcon) and centrifuged mildly, the cells were resuspended with PBS(-). Further the suspended solution was filtered through 40 μ m of Cell Strainer (Falcon); the re-passed solution was centrifuged and resuspended to remove debris including dead cells as much as possible; and the suspended solution was observed under a fluorescence microscope.

[0117]

The results were shown in Fig. 25. Fig. 25 shows A/A, D/D, and D/A images, and the corresponding phase contrast image of IL-2 expression-induced and -uninduced cells. In Fig. 25, two sets of A/A, D/D, D/A images, and the corresponding phase contrast images for each pair of probes were shown. One to three cells out of 20-22 cells were D/A-positive cells in the visual field for IL-2 expression-induced cells, suggesting that the donor probe and the acceptor probe adjacently hybridize to IL-2 mRNA. On the other hand, no D/A-positive cells were

observed for IL-2 expression-uninduced cells.

[0118]

(11) Selective separation of cells which have expressed IL-2 genes by flow cytometry

Utilizing the differenciated intensities of FRET-fluorescence between IL-2 expression-induced and -uninduced cells based on the specific hybrid formation among the donor probe, the acceptor probe, and IL-2 mRNA, it was attempted to separate IL-2 expressing cells from non-expressing cells as follows.

[0119]

The cell suspensions of IL-2 expression-induced cells prepared as described in (9) and those of -uninduced cells were mixed with the ratios of 100:0, 0:100, 50:50, and 20:80, respectively. Then, 0.9 ml of the mixture was put into a cuvette; 16.2 nM (final concentration; 18.0 μ M) of Bodipy493/503-labeled donor probe IL-2 342-356(D) and 14.7 nmol (final concentration; 16.4 μ M) of Cy5-labeled acceptor probe IL-2 357-371(A) were added. The mixture with the probes was pulsed as described in (9). Live cells were collected and were applied to a flow cytometer, an experimental device for flow cytometry (FACSCalibur, BECTON DICKINSON Inc.).

[0120]

At a position in flow path, the excitation light for an energy donor fluorescence dye (Bodipy493/503) was irradiated to the cells. Relative fluorescence intensity emitted from acceptor fluorescence dye (Cy5), representing FRET fluorescence based on the hybridization was measured together with relative intensity of Bodipy493/503. Each cell was

plotted as a dot in a diagram with the X-axis, the intensity of Bodipy493/503 (FL1-Height) and Y-axis, that of Cy5 (FL3-Height). Among these plots, a group of dots with the highest value of FL3-Height representing a group of fluorescing cells based on hybridization, was designated as R2. On the other hand, a group of dots, representing the cell size (FSC-Height; forward-scattering light) as well as the complexity in the intrastructure (SSC-Height; side-scattering light) as typical human lymphoid cells was designated as R1 according to the reference value (FACSCalibur Training Manual, BECTON DICKINSON).

[0121]

The obtained dot-plots were shown in Figs. 26-33. Fig. 26 shows a dot plot for FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 100 to 0. Fig. 27 shows a dot plot of the cells with the same mixing ratio based on FL1-Height and FL3-Height as in Fig. 26. Fig. 28 shows a dot plot of the cells based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 0 to 100. Fig. 29 shows a dot plot of the cells based on the FL1-Height and FL3-Height with the same mixing ratio as in Fig. 28. Fig. 30 shows a dot plot of the cells based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 50 to 50. Fig. 31 shows a dot plot of the cells based on FL1-Height and FL3-Height with the same ratio as in Fig. 30. Fig. 32 shows a dot plot of the cells based on FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 20 to 80. Fig. 33 shows a dot plot of the cells based on FL1-Height and FL3-Height with the same mixing ratio as in Fig. 32.

[0122]

A cell group belonging to both R1 and R2 was collected by a cell sorting function (a cell sorter). Some of the collected cell group was applied to FACSCalibur and detected as similar dot-plots in order to confirm that the group was still fluorescently labeled as desired. The obtained dot-plots were shown in Figs. 34-39. Fig. 34 shows a dot plot for FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 100 to 0. Fig. 35 shows a dot plot of the cells based on FL1-Height and FL3-Height with the same mixing ratio as in Fig. 34. Fig. 36 shows a dot plot for FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 50 to 50. Fig. 37 shows a dot plot of the cells based on FL1-Height and FL3-Height with the same mixing ratio as in Fig. 36. Fig. 38 shows a dot plot for FSC-Height and SSC-Height when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 20 to 80. Fig. 39 shows a dot plot of the cells based on FL1-Height and FL3-Height with the same mixing ratio as in Fig. 38.

[0123]

The comparison of Fig. 27, Fig. 31, and Fig. 33 revealed that the proportion of dots representing the cell group belonging to R2 to the entire number of dots decreased in relation to the decrease in the mixing ratio of IL-2 expression-induced cells from 100, 50 to 20 %, while the value of FL3-Height was totally background level regarding IL-2 expression-uninduced cells even when the same donor and acceptor probes were introduced to the cells (Fig. 29). As most of the sorted-out cells, selectively separated and collected cells, belonged to both R1 and R2, IL-

2 expression-induced cells were found to be collected as the live cells emitting considerable FRET-fluorescence (see Fig. 34-39).

[0124]

(12) Comparison of cell groups before and after flow cytometry by fluorescence microscopy

Some of the cells before and after flow cytometry were transferred to glass-bottomed dishes, and ratios of fluorescing cells of D/A, A/A, and D/D of the cells in the entire visual field were examined.

[0125]

The results were shown in Figs. 40-46. Figs. 40, 42, 44, and 46 show images of the cells before flow cytometry; fluorescence images of acceptor dyes based on FRET representing hybrid formation among IL-2 mRNA, donor probes and acceptor probes (D/A image), fluorescence images of donor dyes by the donor-excitation representing the presence of donor probes in the cells (D/D image), fluorescence images of acceptor dyes by the acceptor-excitation representing the presence of acceptor probes in the cells (A/A image), and the corresponding phase contrast images. Figs. 41, 43 and 45 show images of the cells after flow cytometry, the cells selectively collected by the cell sorting function. The A/A, D/A, and D/D fluorescence images and the corresponding phase contrast images are shown in these figures as in Fig 40,42, 44 and 46. In Figs. 40-46, arrows indicating some cells are shown to align the positions of cells between the fluorescence images and the phase contrast images.

[0126]

Cells in Figs. 40 and 41 were the mixture of IL-2 expression-induced and -uninduced live cells with the ratio of 100 to 0; cells in Figs.

42 and 43 were those with the ratio of 50 to 50. Cell groups in Figs. 34 and 35 were those with the ratio of 20 to 80; and cell groups in Fig. 46 were those with the ratio of 0 to 100.

[0127]

In Fig. 40, the description of 20 cells in the phase contrast micrograph means that there were 20 cells in the entire visual field. The descriptions of 5 cells, 4 cells and 7 cells in A/A, D/A and D/D images represent that the numbers of the cells emitting A/A-, D/A-, and D/D-fluorescence were 5, 4, and 7, respectively.

[0128]

In Fig. 42, the descriptions of 20 cells, 3 cells, 1 cell and 10 cells mean that the number of the cells in the entire visual field was 20 and the numbers of cells emitting A/A-, D/A-, and D/D-fluorescence were 3, 1, and 10 respectively. In Fig. 44, the descriptions of 36 cells, 7 cells, 1 cell and 20 cells mean that the number of the cells in the entire visual field was 36 and the numbers of cells emitting A/A-, D/A-, and D/D-fluorescence were 7, 1, and 20, respectively. In Fig. 46 where IL-2 expression-uninduced cells are contained, the descriptions of 21 cells, 2 cells, 0 cell and 3 cells mean that the number of the cells in the entire field was 21 and the numbers of cells emitting A/A-, D/A-, and D/D-fluorescence were 2, 0, and 3 respectively.

[0129]

Thus, as the ratio of IL-2 expression-induced cells was decreased from 100, 50, to 20 %, it was found that the ratio of D/A-fluorescent cells possessing fluorescently labeled IL-2 mRNA to the cells in the entire visual field decreased.

[0130]

Fig. 41 shows fluorescence images of the cells selectively separated from the mixture of IL-2 expression-induced and -uninduced live cells with the ratio of 100 to 0. The description of 7 cells in the phase contrast micrograph means that 7 cells exist in the entire visual fields. The descriptions of 7 cells, 7 cells, and 7 cells in the A/A, D/A and D/D images represent that all 7 cells in the field were emitting A/A-, D/A- and D/D-fluorescence.

[0131]

In Fig. 43, the descriptions of 6 cells in the phase contrast image and in all the A/A, D/A and D/D images represent that the number of the cells in the entire field was 6 and that all the cells were emitting A/A-, D/A-, and D/D-fluorescence. Similarly in Fig. 45, all the descriptions of 5 cells in the phase contrast image and in all the A/A, D/A and D/D images represent the number of the cells in the entire field was 5 and that all the cells were emitting A/A-, D/A-, and D/D-fluorescence.

[0132]

The comparison between Figs. 40 and 41, between Figs. 42 and 43, and between Figs. 44 and 45 revealed that only the cells in which IL-2 mRNA was fluorescently labeled could be selectively separated by the cell sorting function of flow cytometry (a cell sorter).

[0133]

<u>(13) Comparison between the cells before and after flow cytometry by in situ hybridization</u>

Some of the cells before and after flow cytometry were transferred to a glass-bottomed dish, and fixed with 4% paraformaldehyde

/PBS (pH7.4) at room temperature for 30 minutes. The ratio of cells carrying IL-2 mRNA (IL-2 mRNA (+)) in the entire visual field was determined by fluorescent in situ Hybridization. First, in this FISH, in order to prevent higher background caused by RNA probes remaining in the cell after the wash-out procedure, IL-2 RNA probes, which were obtained by the fragmentation of a full-length anti-sense IL-2 RNA synthesized according to the method in (7) (c), were used for hybridization experiments.

[0134]

A full-length anti-sense IL-2 RNA, 10 μ g, were dissolved in 100 μ l of alkaline solution (42 mM $NaHCO_3$, 63 mM Na_2CO_3, 5 mM DTT), incubated at 60 °C for 10-15 minutes. Then, 10 μ l of 3 M sodium acetate and 350 μ l of ethanol were added to precipitate the RNA probes. After allowing to stand at –20 °C for 30 minutes, they were and centrifuged at 16 krpm for 20 minutes. The obtained precipitates were washed with 70% ethanol and dried. The precipitates were dissolved in 50 μ l of RNase-free sterile distilled water to prepare an alkaline denatured RNA probe solution for IL-2 RNA.

[0135]

The cells fixed at the bottom of the dish were washed with PBS(-) three time and treated with 0.1% Triton X-100/PBS solution at room temperature for 5 minutes to permeabilize the cells, and the permeabilized cells were washed with PBS(-) three times and treated with 0.2N HCl at room temperature for 10 minutes. Washing the monolayer cells with PBS(-), they were incubated with 1 μ g/ml of proteinase K/ PBS solution for 5 minutes at 37 °C. After the monolayer cells were washed with PBS(-), they was fixed again with 4% of parafromaldehyde / PBS (pH 7.4) for 30

minutes. The fixed cells were washed twice with 2 mg/ml of glycine/PBS for 15 minutes, and treated with 50% deionized formamide/2 x SSC solution (solution A, described hereunder) for 30 minutes; the hybridization solution (50% deionized formamide, 5 x denhardt, 2 x SSC, alkaline denatured probes for IL-2 RNA (1 μ g/ml)) was prepared, denatured at 90 °C for 10 minutes, and then the monolayer cells were ice-cooled. Adding 100 μ l of the hybridization solution to the cells, they were incubated at 45 °C overnight.

[0136]

The monolayer cells after the hybridization were washed twice with solution A for 5 minutes at 45 °C, then washed with solution B (10 M Tris · HCl(pH8.5),500 mM NaCl) for 5 minutes, and treated with 20 μ g/ml of RNase A /solution B (pretreated at 90 °C for 10 minutes) at 37 °C for 20 minutes. They were washed with solutions A at 45 °C for 30 minutes and C (50% of deionized formamide/1 x SSC) at 45 °C for 30 minutes and with solution C at room temperature for 20 minutes. After washing with Buffer 1 [100 mM maleic acid, 150 mM NaCl (pH7.5)] twice for 5 minute, they were treated with Buffer 2 [1% Blocking Reagent(Boehringer Mannheim Inc.) in Buffer 1] at room temperature for 20 minutes.

[0137]

After the monolayer cells were washed with Buffer I twice, FITC-labeled anti-DIG antibodies (Fab, diluted with Buffer 2 by 100 times, protein level: about 1 μ g/ml) was added, incubated for at least 30 minutes, washed with PBS(-) three times. The cells were observed under a fluorescence microscope; and the ratio of cells carrying IL-2 mRNA to the total cells in the visual field was determined.

[0138]

Figs. 47 and 48 show fluorescence micrographs when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 100 to 0. Fig. 47 shows micrographs before flow cytometry. Fig. 48 shows micrographs after flow cytometry. Fig. 49 shows fluorescence micrographs before flow cytometry when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 0 to 100. Figs. 50 and 51 show fluorescence micrographs when the mixing ratio of IL-2 expression-induced cells to -uninduced cells was 50 to 50. Fig. 50 shows micrographs before flow cytometry and Fig. 51 shows those after flow cytometry. Figs. 52 and 53 show fluorescence micrographs when the mixing ratio of IL-2 expression-induced cells to -uninduced cell was 20 to 80. Fig. 52 shows fluorescence micrographs before flow cytometer, and Fig. 53 shows those after flow cytometry.

[0139]

The figures in the micrographs represent the numbers of fluorescing cells per the number of total cells in the entire visual field. In Fig. 47, "48/48" represents that all of the 48 cells were fluorescing cells in the image, suggesting that all the cells were IL-2 mRNA carrying cells. On the contrary, "0/32" in Fig. 49 represents that there were no IL-2 mRNA carrying cells out of 32 cells. In Fig. 50, "18/35" represents that 18 cells possess IL-2 mRNA out of 35 cells. In Fig. 52, "8/39" represents that 8 cells possess IL-2 mRNA out of 39 cells. These results are well consistent with the fact that IL-2 expression-induced and -uninduced cells were mixed with the ratios of 50 to 50 in Fig. 50 and 20 to 80 in Fig. 52. On the other hand, the figures in Fig. 48, Fig. 51, and Fig. 53 were 9/9, 7/7, and

8/8, respectively, suggesting that IL-2 mRNA carrying cells were condensed from 20-50% to 100% throughout flow cytometry.

[0140]

(14) Separation method based on fluorescence intensities of live cells expressing specific genes

Table 8 is a summarized result of (11)-(13) to show the effect of this separation method utilizing the differenciated intensities based on the fluorescent labeling of IL-2 mRNA. The cells carrying IL-2 mRNA which were condensed from 20-50% to 100% by the separation method utilizing the difference in fluorescence intensities. That is, all the live cells carrying IL-2 mRNA were selectively separated from the live cell group containing, IL-2 mRNA carrying cells.

[0141]

Table 8

Mixing Ratios		Flow Cytometry			
		Before		After	
IL-2 expression		FRET (D/A) Positive Cells(%)	Cells carrying IL-2 mRNA (%)	FRET (D/A) Positive Cells(%)	Cells carrying IL-2 mRNA (%)
Induced	uninduced				
100	0	20	100	100	100
0	100	0	0	-	-
50	50	5	51.4	100	100
20	80	2.8	20.5	100	100

[0142]

(15) Separation of lymphocytes from human peripheral blood

After sampling 200 ml of blood from a healthy adult and adding heparin thereto at a final concentration of 10 U/ml, it was mixed

with a two-fold volume of 3% dextrin in PBS (phosphate buffered saline) in a 50 ml centrifugation tube (Falcon 2070), and the mixture was allowed to stand at room temperature for 15 minutes to precipitate erythrocytes. 21.5 ml of the supernatant was gently superposed onto a 15 ml of Ficoll pack (Pharmacia) and subjected to centrifugation at 490 x g (1,600 rpm) for 30 minutes. The lymphocyte layer observed as white suspended matter in the supernatant was collected with a pipette. After mixing the lymphocyte layer with a three-fold volume of HBSS (Hanks' Balanced Salt Solution, GIBCO BRL) in a 50 ml centrifugation tube (Falcon 2070) and centrifuging at 1,200 rpm for 10 minutes, the lymphocyte precipitate was washed twice with 30 ml of PBS(-) and suspended to a cell density of about 2.5×10^7 cells/ml.

[0143]

(16) Separation and fluorescent antibody staining of helper T cells (CD4+ cells) from peripheral blood lymphocytes

Separation of CD4+ cells from peripheral blood lymphocytes was performed using Human CD4+ cell Recovery Column Kit (CEDARLANE Laboratories, Ltd.) was used for separation of the CD4+ cells, by a separation procedure according to the protocol provided with the kit.

[0144]

A CD4+ cell separating column (CEDARLANE) was set in a column stand and the glass beads in the column were equilibrated with 15 ml of PBS(-), with only a slight amount of the PBS(-) remained above the beads. To coat these beads with goat anti-human IgG (H+L) and goat anti-mouse IgG (H+L), the powder of Column Reagent (CEDARLANE) was dissolved in 1-1.5 ml of PBS(-), and applied to the column and allowed to flow till

only a slight amount remained above the beads, and this was allowed to stand at room temperature for 1-8 hours.

[0145]

To neutralize CD8+ cells in the cell sample with CD8-specific antibodies (mouse anti-human CD8), the powder of Cell Reagent (CEDARLANE) was dissolved in 1.5 ml of PBS(-), and the total amount of this Cell Reagent solution was mixed with 3.5-4.5 ml of the lymphocyte suspension prepared in (15) above in a 50 ml centrifugation tube (Falcon 2070) and incubated on ice for at least 30 minutes. After adding 15 ml of PBS(-) to the lymphocyte suspension, it was centrifuging at 4° C, 200 x g (approximately 1,200 rpm) for 5-10 minutes, the supernatant was removed by pipetting.

[0146]

The resulting cell precipitate was washed again with 15 ml of PBS(-) and suspended to a cell density of about 5×10^7 cells/ml PBS (-). The beads equilibrated with PBS(-) in a column as described above were washed with 20 ml of PBS(-) adjusting the flow rate to 6-8 drops/min (1 drop/8 sec), and then the lymphocyte suspension was poured down over the column beads, the eluate was collected in a 15 ml tube (Falcon), and the pouring was stopped when a slight amount of the suspension remained above the beads. PBS (-) was further poured onto the beads, and 10-15 ml of eluate was collected. The obtained eluates were centrifuged at 4° C at 1,200 rpm for 10 minutes, the supernatant was removed by pipetting, and the obtained precipitate was suspended to a cell density of 1.0×10^7 cells/ml in PBS(-) containing 10% fetal bovine serum (FBS).

[0147]

In order to determine the ratio of CD4+ T cells in the lymphocyte suspensions, 20 µl of anti-CD4/CD8 antibody (Simultest (Leu-3A/2a), Becton Dickinson) and a control antibody (Simultest control, Becton Dickinson) was mixed with 50 µl of the cell suspension in a 2 ml microtube and incubated on ice in the dark for 30-45 minutes, and the mixture was subjected to fluorescent staining of the CD4 and CD8 on the cell surface. The mixture was diluted with 2 ml PBS(-), mixed with vortexing, centrifuged at 300 x g for 5 minutes, and then the supernatant was removed by pipetting or aspiration. The precipitate was suspended in 1 ml of PBS(-), the lymphocyte suspension was applied to a flow cytometer (FACSCalibur, Becton Dickinson) and analyzed for CD4 and CD8. The results confirmed abundant CD8+ cells among the peripheral blood lymphocytes before the column separation (Fig. 56). In contrast, although about 50% of the obtained cells from the CD4+ cell separation procedure described above was CD4 negative or weakly positive, there was no contamination of CD8+ cells (Fig. 59). Accordingly the purified cells was used as CD4 positive cells for further experiments.

[0148]

(17) Activation of helper T cells (CD4+ cells)

After adding 1 μ g/ml (final concentration) of ionomycin (SIGMA) and 30 nM PMA (SIGMA) to the CD4 positive cells obtained in (16) (cell density: 1.0×10^7 cells/ml), the mixture was incubated for 2 hours at 37° C in the presence of 5% CO_2.

[0149]

(18) Fluorescent labeling of intracellular IL-2 mRNA of helper T cells (CD4+ cells)

0.9 ml of the activated CD4+ cell suspension obtained in (17) was transferred to an electroporation cuvette (Gene Pulser specialized cuvette (electrode spacing = 0.4 cm), BIO-RAD), and after adding 5.4 nmol (final concentration: 6.0 μ M) of Bodipy493-503-labeled donor probe IL-2 342-356(D) and 5.3 nmol (final concentration: 5.86 μ M) of Cy5-labeled acceptor probe IL-2 357-371(A), a pulse was applied to the cells at 250 V, 975 μ F. The cell suspension was filtered through 70 μ m Cell Strainer (Falcon), and after moderate centrifugation the cells were resuspended with PBS(-). The cell suspension was then filtered through 40 μ m Cell Strainer (Falcon) to remove as much of the dead cell-containing debris as possible, centrifuged and the precipitates was resuspended with PBS(-). Some of the cells was transferred to a cover glass chamber (Lab-Tek II Chambered Coverglass #155409, NUNC Co.) and observed under a fluorescence microscope to examine the ratio of fluorescing cells among all the cells in the visual field.

[1] A/A {fluorescence of A (acceptor dye) emitted from cells when the excitation light for A was irradiated to the cells}

[2] D/A {fluorescence of A emitted from cells when the excitation light for D (donor dye) was irradiated to the cells; FRET fluorescence}

[3] D/D {fluorescence of D emitted from cells when the excitation light for D was irradiated to the cells}

[0150]

Three cells in the entire visual field (25 cells) were observed emitting D/A fluorescence, indicating specific fluorescent labeling of IL-2

mRNA based on hybridization between the mRNA and the donor and acceptor probes. This result suggested that TH1 cells present at about 12% in activated CD4+ cells (Fig. 60).

[0151]

(19) Selective separation of TH1 by flow cytometry from activated CD4+ cells

The difference in fluorescence intensity (between IL-2 mRNA carrying and non-carrying cells) produced by FRET fluorescence caused by hybridization of IL-2 mRNA with the donor and acceptor probe in live cells was utilized in the following attempt to selectively separate TH1.

[0152]

A suspension of the fluorescent IL-2 probes-introduced cells prepared in (18) was applied to a flow cytometer (FACSCalibur). At a position in flow path, the excitation light of a donor fluorescent dye (Bodipy) was irradiated to the cells to detect FRET fluorescence emitted from acceptors (Cy5) caused by the hybridization, and then relative fluorescence intensity of Cy5 or Bodipy was shown as FL3-Height or FL1-Height, respectively in a dot-plots diagram. Among these plots, a group of cells with the highest value of FL3-Height was designated as R2 (Fig. 63).

[0153]

On the other hand, a group of typical human lymphocytes in the points of cell size (FSC-Height; forward-scattering light) as well as the complexity in the intrastructure (SSC-Height; side-scattering light) was designated as R1 according to the reference value (FACSCalibur Training Manual, BECTON DICKINSON). Cells belonging to both R1 and R2 were selectively separated using a cell sorting function. The separated cells

were again applied to the FACSCalibur to confirm that they were the objective cells. The majority of the sorted out cells by the cell sorting function belonged to both R1 and R2, indicating that the cells with fluorescently labeled IL-2 mRNA had been separated out as ones emitting considerable FRET fluorescence (Fig. 65). In contrast, CD4+ cells introduced no fluorescent probes were detected as dots near at the base line of FL3-Height with only weak fluorescence at FL1-Height (Fig. 67). These results indicated that cells with specifically fluorescent labeled IL-2 mRNA can be clearly distinguished on dot plots.

[0154]

Some of the sorted out cells were then observed under a fluorescence microscope in the same manner as (18). All 8 cells in the entire visual field emitted D/A (FRET fluorescence), suggesting that IL-2 mRNA was fluorescently labeled in all the sorted out cells (Fig. 61). Comparing the result above with that before cytometry in (18) for the ratios of CD4+ cells with fluorescent labeled IL-2 mRNA, it was suggested that TH1 had been selectively separated by the cell sorting function.

[0155]

(20) Fluorescent labeling of intracellular IL-4 mRNA of helper T cells (CD4+ cells)

0.9 ml of the activated CD4+ cell suspension obtained in (17) was transferred to an electroporation cuvette (BIO-RAD) in the same manner as (18), and after adding 15.1 nmol (final concentration: 16.8 μ M) of Bodipy493-503-labeled donor probe IL-4 265-279 and 13.6 nmol (final concentration: 15.1 μ M) of Cy5-labeled acceptor probe IL-4 280-294, a pulse was applied to the cells at 250 V, 975 μ F. In the same manner as

(18), the cell suspension was filterated through 70 μm Cell Strainer (Falcon), and after moderate centrifugation the cells were resuspended with PBS(-). To remove as much of the dead cell-containing debris as possible, the suspension was then filtered through 40 μm Cell Strainer (Falcon), centrifuged and resuspended and then the cells were transferred to a cover glass chamber (NUNC) and observed under a fluorescence microscope to examine the ratio of A/A, D/A and D/D fluorescing cells among the total cells in the visual field, in the same manner as (18). Two cells in the entire visual field (41 cells) were emitting D/A fluorescence, indicating specific fluorescent labeling of IL-4 mRNA based on hybridization between the mRNA and the donor and acceptor probes (Fig. 68). This result suggested that TH2 is present at about 5% in activated CD4+ cells.

[0156]

(21) Selective separation of TH2 by flow cytometry from activated CD4+ cells

The difference in fluorescence intensity (between IL-4 mRNA carrying and non-carrying cells) caused by FRET fluorescence based on hybridization of IL-4 mRNA with the donor and acceptor probe in live cells was utilized to selectively separate TH2.

[0157]

A suspension of the fluorescent probes-introduced cells obtained in (20) was applied to a flow cytometer (FACSCalibur). The excitation light of a donor dye (Bodipy) was irradiated to the cells to detect FRET fluorescence emitted from acceptors (Cy5) based on the hybridization in the same manner as (19), and then relative fluorescence intensity of

Bodipy or Cy5 was shown as FL1-Height or FL3-Height, respectively in dot-plots diagram. Among these plots, a group of cells with the highest value of FL3-Height was designated as R2 (Fig. 71).

[0158]

On the other hand, a group of typical human lymphocytes in the points of cell size (FSC-Height; forward-scattering light) as well as the complexity in the intrastructure (SSC-Height; side-scattering light) was designated as R1 (Fig. 70). Cells belonging to both R1 and R1 was selectively separated using a cell sorting function. The separated cells were again applied to FACSCalibur and examined to confirm that they were the objective fluorescently labeled cells. The majority of the sorted out cells by the cell sorting function belonged to both R1 and R2 (Figs. 72 and 73), indicating that the cells with fluorescent labeled IL-4 mRNA had been separated out as ones emitting considerable FRET fluorescence.

[0159]

Some of the sorted-out cells were then observed under a fluorescence microscope in the same manner as (20). All 7 cells in the entire visual field emitted D/A (FRET fluorescence), suggesting that IL-4 mRNA was fluorescently labeled in all the sorted-out cells (Fig. 69). Comparing the result above with that before cytometry in (20) for the ratios of CD4+ cells with fluorescent labeled IL-4 mRNA, it was suggested that TH2 had been selectively separated by the cell sorting function.

[0160]

<u>(22) Induction of TH2 among helper T cells (CD4+ cells)</u>

A balance between TH1 and TH2 is maintained in healthy bodies, and it is believed that this balanced relationship supports homeostasis in

immune system of the body. Conversely, disruption of the balance between TH1 and TH2 is a cause of onset of numerous immune diseases. While there is no doubt that factors causing disruption in this balance in the body are the sources responsible for such diseases, the mechanisms leading to their onset are too complicated to reconstitute in vitro. Nevertheless, several approaches to mimic the disruption have long been performed as follows. When TH2 is dominant over TH1, it promotes excess production and secretion of immunoglobulins (antibody molecules) by B cells as their humoral immune function. In this unbalanced state, the production of autoantibodies (react with self components to cause tissue damage) is induced to result in autoimmune diseases.

[0161]

An approach to mimic this condition by artificially preparing a TH2-dominant helper T cell group is one utilizing the property of TH2, "IL-4 autocrine", i.e., TH2 activates itself by a cytokine (IL-4) which it produces", whereby a helper T cell group is treated with a high concentration of IL-4 to induce differentiation to TH2 (Openshaw, P. et al., J. Exp. Med. 182(5), 1357, 1995). Here, 20 ng/ml (final concentration) of human recombinant IL-4 (Genzyme) was added with 1 μ g/ml ionomycin and 30 nM PMA as described in (17) to CD4+ cells obtained in (16), and the cells were incubated at 37° C for 2 hours.

[0162]

<u>(23) Fluorescent labeling of TH1 intracellular IL-2 mRNA among TH2-dominant helper T cells</u>

TH1 cells were selectively separated and obtained from TH2-dominant helper T cell group in which TH1 is sparsely present as follows.

0.9 ml of the TH2-induced CD4+ cell suspension obtained in (22) was transferred to an electroporation cuvette (BIO-RAD) in the same manner as (18). After adding 5.4 nmol (final concentration: 6.0 μ M) of Bodipy493-503-labeled donor probe IL-2 342-356 and 5.3 nmol (final concentration: 5.86 μ M) of Cy5-labeled acceptor probe IL-2 357-371, a pulse was applied to the cells at 250 V, 975 μ F. In the same manner as (18), the debris containing most of the dead cells was removed from the cell suspension and then some of the cells were transferred to a cover glass chamber (NUNC) and observed under a fluorescence microscope to examine the ratio of A/A, D/A and D/D fluorescing cells among the cells in the entire visual field. One cell in the entire visual field (23 cells) was emitting D/A fluorescence, indicating the presence of a cell with specific fluorescent labeling of IL-2 mRNA (Fig. 74). Comparing this result with that in (18), it was suggested that TH1 is reduced to approximately 4% in the TH2-dominant CD4+ cells from 12% in the unselectively activated CD4+ cells (Fig. 60).

[0163]

(24) Selective separation of TH1 by flow cytometry from TH2-dominant helper T cell group

The difference in fluorescence intensity (between IL-2 mRNA carrying and non-carrying cells) caused by FRET fluorescence based on hybridization of IL-2 mRNA with the donor and acceptor probe in live cells was utilized to selectively separate TH1.

[0164]

A suspension of the fluorescent probes-introduced cells obtained in (23) was applied to a flow cytometer (FACSCalibur). The excitation light

of a donor dye (Bodipy) was irradiated to the cells to detect FRET fluorescence emitted from acceptors (Cy5) based on the hybridization in the same manner as (19), and then relative fluorescence intensity of Bodipy or Cy5 was shown as FL1-Height or FL3-Height, respectively in dot-plots diagram. Among these plots, a group of cells with the highest value of FL3-Height was designated as R2 (Fig. 77). On the other hand, a group of typical human lymphocytes in the points of cell size (FSC-Height; forward-scattering light) as well as the complexity in the intrastructure (SSC-Height; side-scattering light) was designated as R1 (Fig. 76). Cells belonging to both R1 and R1 was selectively separated using a cell sorting function.

The separated cells were again applied to the FACSCalibur and examined to confirm that they were the objective fluorescently labeled cells. The majority of the sorted out cells by the cell sorting function belonged to both R1 and R2 (Figs. 78 and 79), indicating that the cells with fluorescent labeled IL-2 mRNA had been separated out as ones emitting considerable FRET fluorescence.

[0165]

Some of the sorted out cells were then observed under a fluorescence microscope in the same manner as (20). All 5 cells in the entire visual field emitted D/A (FRET fluorescence), suggesting that IL-2 mRNA was fluorescently labeled in all the sorted-out cells (Fig. 75). Comparing the result above with that before cytometry in (23) for the ratios of CD4+ cells with fluorescent labeled IL-2 mRNA, it was suggested that TH1 had been selectively separated by the cell sorting function.

[0166]

(25) Induction of TH1 among helper T cells (CD4+ cells)

In contrast to (22), it is known that when TH1 is dominant over TH2, it provokes a chronic disease such as tuberculoid leprosy (Mitra, D.K. et al. Int. Immunol. 11(11), 1801, 1999). An approach to mimic this condition by artificially preparing a TH1- dominant helper T cell group is one whereby a helper T cell group is treated with the TH1-activating cytokine IL-12 and with specific antibodies for IL-4 to neutralize and inactivate IL-4 in the extracellular fluid to induce TH1 (Openshaw, P. et al., J. Exp. Med. 182(5), 1357, 1995).

[0167]

Here, 10 ng/ml (final concentration) of human recombinant IL-12 (Genzyme) and 10 μg/ml anti-human IL-4 mouse monoclonal antibody (Genzyme) were added with 1 μg/ml ionomycin and 30 nM PMA as described in (17) to CD4+ cells obtained in (16), and the cells were incubated at 37° C for 2 hours.

[0168]

(26) Fluorescent labeling of TH2 intracellular IL-4 mRNA among TH1-dominant helper T cells

TH2 cells were selectively separated and obtained from TH1-dominant helper T cell group in which TH2 is sparsely present as follows. 0.9 ml of the TH1-induced CD4+ cell suspension obtained in (25) was transferred to an electroporation cuvette (BIO-RAD) in the same manner as (18). After adding 15.1 nmol (final concentration: 16.8 μM) of Bodipy493-503-labeled donor probe IL-4 265-279(D) and 13.6 nmol (final concentration: 15.1 μM) of Cy5-labeled acceptor probe IL-4 280-294(A), a pulse was applied to the cells at 250 V, 975 μF.

[0169]

In the same manner as (18), the debris containing most of the dead cells was removed from the cell suspension and then some of the cells were transferred to a cover glass chamber (NUNC) and observed under a fluorescence microscope to examine the ratio of A/A, D/A and D/D fluorescing cells among the cells in the entire visual field. One cell in the entire visual field (42 cells) was emitting D/A fluorescence, indicating the presence of a cell with specific fluorescent labeling of IL-2 mRNA (Fig. 80). Comparing this result with that in (18), it was suggested that TH2 is remained at approximately 2% in the TH1-dominant CD4+ cells.

[0170]

(27) Selective separation of TH2 by flow cytometry from TH1-dominant helper T cell group

The difference in fluorescence intensity (between IL-4 mRNA carrying and non-carrying cells) caused by FRET fluorescence based on hybridization of IL-4 mRNA with the donor and acceptor probe in live cells was utilized to selectively separate TH2.

[0171]

A suspension of the fluorescent probes-introduced cells obtained in (26) was applied to a flow cytometer (FACSCalibur). The excitation light of a donor dye (Bodipy) was irradiated to the cells to detect FRET fluorescence emitted from acceptors (Cy5) based on the hybridization in the same manner as (19), and then relative fluorescence intensity of Bodipy or Cy5 was shown as FL1-Height or FL3-Height, respectively in dot-plots diagram. Among these plots, a group of cells with the highest value of FL3-Height was designated as R2 (Fig. 83). On the other hand, a

group of typical human lymphocytes in the points of cell size (FSC-Height; forward-scattering light) as well as the complexity in the intrastructure (SSC-Height; side-scattering light) was designated as R1 (Fig. 82). Cells belonging to both R1 and R1 was selectively separated using a cell sorting function.

The separated cells were again applied to FACSCalibur and examined to confirm that they were the objective fluorescently labeled cells. The majority of the sorted-out cells by the cell sorting function belonged to both R1 and R2 (Figs. 84 and 85), indicating that the cells with fluorescent labeled IL-4 mRNA had been separated out as ones emitting considerable FRET fluorescence.

[0172]

Some of the sorted-out cells were then observed under a fluorescence microscope in the same manner as (20). All the cells in the entire visual field emitted D/A (FRET fluorescence), suggesting that IL-4 mRNA was fluorescently labeled in all the sorted-out cells (Fig. 81). Comparing the result above with that before cytometry in (26) for the ratios of CD4+ cells with fluorescent labeled IL-4 mRNA, it was suggested that TH2 had been selectively separated by the cell sorting function.

[0173]

<u>(28) Detection of IL-2 mRNA or IL-4 mRNA carrying cells before flow cytometry by in situ hybridization</u>

Some of the cells before flow cytometry (cell sorter separation) were transferred to a cover glass chamber (Lab-Tek II Chambered Coverglass #155409, NUNC Co.), and after fixing the cells with 4% paraformaldehyde/PBS (pH 7.4) at room temperature for 30 minutes, the

FISH (Fluorescence in situ Hybridization) method described below was used to determine the ratio of cells carrying IL-2 mRNA or IL-4 mRNA (IL-2 mRNA(+) or IL-4 mRNA(+)) present among the cells in the entire visual field.

[0174]

First, a digoxigenin (hereunder, DIG)-labeled RNA probe for IL-2 RNA to detect intracellular IL-2 mRNA of the fixed cells was synthesized using a DIG RNA Labeling Kit (Boehringer Mannheim) according to the protocol of the kit manual. 10 μg of recombinant plasmid (pTCGF#2) constructed for human IL-2 RNA synthesis by the method described in (2A) was completely linearized by EcoRI digestion, and the linearized DNA in the obtained DNA solution was extracted with phenol/chloroform for denaturation and removal of protein and then purified by ethanol precipitation, to prepare a template for RNA probe synthesis. This template DNA (5 μg) was mixed with 1.8 mM ATP, 0.9 mM CTP, 0.7 mM GTP, 1.1 mM UTP and 0.58 mM UTP (DIG-labeled) in the presence of T7 RNA polymerase and incubated at 37° C for 2 hours. After DNaseI solution was added to the solution and incubated for 10 minutes to degrade the template DNA, a 1/10 volume of 5 M sodium acetate and an equal volume of isopropanol were added to this reaction solution and centrifuged at 15,000 g x 15 min to recover the synthesized RNA a precipitate. The precipitate was dissolved in RNase-free sterile distilled water.

[0175]

Secondly, a DIG-labeled RNA probe for IL-4 RNA to detect intracellular IL-4 mRNA of the fixed cells was synthesized using a DIG

RNA Labeling Kit (Boehringer Mannheim) in the same manner as the IL-2. A recombinant plasmid (phIL-4#9) constructed for human IL-4 RNA synthesis by the method described in (2B) was linearized by complete digestion with SmaI, and the linearized DNA was treated with phenol/chloroform and then purified by ethanol precipitation, to prepare a template for RNA probe synthesis. This template DNA (5 μg) was mixed with 1.2 mM ATP, 1.0 mM CTP, 1.1 mM GTP, 0.8 mM UTP and 0.5 mM UTP (DIG-labeled) in the presence of T7 RNA polymerase and incubated at 37° C for 2 hours. After DNaseI solution was added and reacted for 10 minutes to degrade the template DNA, a 1/10 volume of 5 M sodium acetate and an equal volume of isopropanol were added to this reaction solution, and centrifuged at 15,000 g x 15 min to recover the synthesized RNA as a precipitate. The precipitate was dissolved in RNase-free sterile distilled water.

[0176]

Thirdly, highly fragmented RNA probes for IL-2 or IL-4 were prepared to use for a hybridization experiment as in (13) because the introduction of the full length IL-2 or IL-4 RNA probe obtained above was thought to cause high background noise due to the remaining non-hybridized RNA probe in the cells. 10 μg of the IL-2 or IL-4 RNA probe was dissolved in 100 μl of an alkaline solution (42 mM $NaHCO_3$, 63 mM Na_2CO_3, 5 mM DTT) and incubated at 60° C for 10-15 minutes. 10 μl of 3 M sodium acetate and 350 μl of EtOH were added to precipitate the RNA. After standing at -20° C for 30 minutes, it was centrifuged at 16 krpm for 20 minutes. The precipitate was washed with 70% ethanol, dried, and dissolved in 50 μl of RNase-free sterile distilled water to prepare an

alkaline denatured IL-2 or IL-4 RNA probe.

[0177]

The cells fixed on the bottom of the chamber were washed 3 times with PBS(-), treated with a 0.1% Triton X-100/PBS solution at room temperature for 5 minutes to permeabilize the cells, and then washed 3 times with PBS(-) and treated with 0.2 N HCl at room temperature for 10 minutes. After washing the cell monolayer with PBS(-), it was incubated for 5 minutes at 37° C with 1 μ g/ml of Proteinase K/PBS solution. After washing the cell monolayer with PBS(-), it was fixed again with 4% paraformaldehyde/PBS (pH 7.4) for 30 minutes. This was washed twice with 2 mg/ml of glycine/PBS (15 minutes per washing) and treated with 50% deionized formaldehyde/2 x SSC solution (hereunder, Soln. A) for 30 minutes to prepare a hybridization solution (50% deionized formaldehyde, 5 x Denhardt, 2 x SSC, alkaline denatured IL-2 or IL-4 RNA probe (1 μ g/ml)). The solution was denatured at 90° C for 10 minutes and cooled on ice. Adding 100 μ l of the solution to the cells, they were incubated for hybridization overnight at 45° C.

[0178]

The cell monolayer after hybridization was washed with Soln. A for 5 minutes at 45° C, and then washed twice with Soln. B (10 mM Tris·HCl (pH 8.5), 500 mM NaCl) (5 min/washing) and treated with 20 mg/ml RNase A/Soln. B (pretreated at 90° C for 10 minutes) at 37° C for 20 minutes. After washing with Soln. A, and then Soln. C (50% deionized formaldehyde/1 x SSC) for 30 minutes each at 45° C, the cells were washed again with Soln. C at room temperature for 20 minutes. After washing with Buffer 1 (100 mM maleic acid, 150 mM NaCl (pH 7.5)) (2 x 5

min), it was subjected to blocking with Buffer 2 (1% Blocking Reagent (Boehringer Mannheim) in Buffer 1) at room temperature for 20 minutes. After washing twice with Buffer 1, FITC-labeled anti-DIG antibody (Fab, diluted with Buffer 2 by 100 times, protein concentration: approximately 1 μ g/ml) was added to the cells and incubated for at least 30 minutes. After washing 3 times with PBS(-), the cells were observed under a fluorescence microscope to examine the ratio of IL-2 mRNA or IL-4 mRNA carrying cells (TH1 or TH2) among the cells in the entire visual field.

[0179]

In the cells used for the experiment of (18) in which IL-2 mRNA was fluorescently labeled for selective separation of TH1, 5 cells were found to carry IL-2 mRNA out of 24 cells and 3 cells out of 43 cells were carrying IL-4 mRNA (Fig. 86). The ratio of IL-2 mRNA carrying cells (20.8%) was somewhat higher than that in (18) (12.0%). This gap is thought to be caused by the difference in the way of probe-introduction, i.e., the fluorescent probes were not introduced to some of TH1 cells in the experiment of (18).

[0180]

In the cells for the experiment of (20) in which IL-4 mRNA was fluorescently labeled for selective separation of TH2, 6 cells were found to carry IL-2 mRNA out of 28 cells and 3 cells out of 42 cells were carrying IL-4 mRNA (Fig. 88). The ratio of IL-4 mRNA carrying cells (7.1%) was slightly higher than that in (20) (4.9%).

[0181]

Furthermore, in the cells used for the experiments of (23) in which IL-2 mRNA was fluorescently labeled for selective separation of TH1 from

a TH2-dominant cell group, 2 cells out of 28 cells and 3 cells out of 35 cells were found to carry IL-2 mRNA, while 8 cells out of 41 cells and 9 cells out of 42 cells were carrying IL-4 mRNA (Fig. 90). The ratio of IL-2 mRNA carrying cells (7.9%) was higher than that in (23) (4.3%). This gap is thought to be caused by the difference in the way of probe-introduction, i.e., the fluorescent probes were not introduced to some of TH1 cells in the experiments of (23).

[0182]

Also, in the cells used for the experiments of (26) in which IL-4 mRNA was fluorescently labeled for selective separation of TH2 from a TH1-dominant cell group, 9 cells out of 24 cells and 11 cells out of 29 cells were found to carry IL-2 mRNA, while 1 cells out of 48 cells and 1 cells out of 41 cells were carrying IL-4 mRNA (Fig. 92). The ratio of IL-4 mRNA carrying cells (2.2%) was equal to that in (26) (2.4%). This consistence between the fluorescence microscope observation results and FISH results for IL-4 mRNA would be due to much the same frequency of probe-introduction, i.e., much the same amount of fluorescent probes were introduced to almost all the TH2 cells. This relatively uniformed introduction is thought to be obtained by the application of electrical pulse to the cells in the presence of a higher concentration of IL-4 fluorescent probe compared with IL-2 probe.

[0183]

<u>(29) Detection of IL-2, γ-IF, TNF-β, IL-4, IL-5 and IL-10 mRNA carrying cells in the cells after flow cytometry (selectively separated with a cell sorter) by in situ hybridization</u>

Some of the cells after flow cytometry (selectively separated with a

cell sorter) obtained in (24) to (27) were transferred to a cover glass chambers (NUNC). After fixing the cells with 4% paraformaldehyde/PBS (pH 7.4) at room temperature for 30 minutes, the FISH method described in the detail in (28) was used to examine the ratios of CD4+ cells having mRNA for the TH1 cytokines IL-2, γ-IF, TNF-β and the TH2 cytokines IL-4, IL-5 and IL-10. Since alkaline denatured RNA probes for IL-2 and IL-4 had already been obtained in (28), DIG-labeled RNA probes to detect mRNA for each of the cytokines other than IL-2 and IL-4 were synthesized using a DIG RNA Labeling Kit (Boehringer Mannheim) according to the protocol of the kit manual.

[0184]

First, for γ-IF, plasmid DNA (pPLc28-HIIF52) including human γ-IF cDNA was extracted and purified using a Plasmid Midi Kit (QIAGEN) from the plasmid-carrying *E. coli* (ATCC#39278) that had been cultured at 28° C. The plasmid was digested with restriction enzymes BamHI and ClaI, and the obtained γ-IF cDNA fragment was linked to the AccI and BamHI restriction site of a pBluescript KS(+) vector for RNA synthesis using DNA Ligation kit version 2 (Takara). The DNA solution was introduced into competent cells of *E. coli* JM109 (Takara) and the recombinant plasmid DNA was extracted and purified from 100 ml culture of the resulting *E. coli* transformants using a Plasmid Midi Kit (QIAGEN). The recombinant plasmid (phγ-IF#1) was digested with restriction enzyme KpnI. After the linearized plasmid DNA was treated with phenol/chloroform, it was purified by ethanol precipitation to prepare a template for RNA probe synthesis. This template DNA (5 μg) was mixed with 1.3 mM ATP, 0.7 mM CTP, 0.8 mM GTP, 0.8 mM UTP and 0.43 mM UTP (DIG-labeled) in the presence

of T7 RNA polymerase and incubated at 37° C for 7 hours. After adding DNaseI to the reaction mixture, it was incubated for 10 minutes to degrade the template DNA. After a 1/10 volume of 5 M sodium acetate and an equal volume of isopropanol were added to the RNA solution, it was centrifuged at 15,000 g x 15 min to recover the synthesized RNA as a precipitate. The precipitate was dissolved in RNase-free sterile distilled water.

[0185]

Secondly, for TNF-β, plasmid DNA carrying human TNF-β cDNA was extracted and purified using a Plasmid Midi Kit (QIAGEN) from 50 ml culture of *E. coli* HILBI37 (ATCC#104607) carrying the plasmid. The plasmid was digested with restriction enzyme BamHI. The linearized plasmid DNA was treated with phenol/chloroform and purified by ethanol precipitation to prepare a template for RNA probe synthesis. The template DNA (5 μg) was mixed with 0.7 mM ATP, 1.4 mM CTP, 1.1 mM GTP, 0.4 mM UTP and 0.24 mM UTP (DIG-labeled) in the presence of T7 RNA polymerase and incubated at 37° C for 6 hours. After adding DNaseI to the reaction mixture, it was incubated for 10 minutes to degrade the template DNA. After adding a 1/10 volume of 5 M sodium acetate and an equal volume of isopropanol to the RNA solution, it was centrifuged at 15,000 g x 15 min to recover the synthesized RNA as a precipitate. The precipitate was dissolved in RNase-free sterile distilled water.

[0186]

Thirdly, for IL-5, 2 μg of lyophilized human IL-5 cDNA-containing plasmid DNA, phIL-5-115.1 (ATCC#59395), was dissolved in 2 μl of sterile distilled water. 1 ng of the plasmid was introduced into competent

cells of *E. coli* JM109 (Takara) and the plasmid DNA was extracted and purified using a Plasmid Midi Kit (QIAGEN) from 50 ml culture of the resulting *E. coli* transformants. The plasmid DNA was digested with restriction enzyme BamHI, and the isolated IL-5 cDNA fragment was linked to the BamHI restriction site of a pBluescript KS(+) vector for RNA synthesis using DNA Ligation kit (Takara).

[0187]

The DNA solution was introduced into competent cells of *E. coli* JM109 (Takara). The recombinant plasmid DNA was extracted and purified from 100 ml culture of the resulting *E. coli* transformants using a Plasmid Midi Kit(QIAGEN). The recombinant plasmid (phIL-5#8) was digested with restriction enzyme NotI. After treating the linearized DNA with phenol/chloroform, it was purified by ethanol precipitation to prepare a template for RNA probe synthesis. This template DNA (5 μg) was mixed with 1.3 mM ATP, 0.7 mM CTP, 0.8 mM GTP, 0.8 mM UTP and 0.42 mM UTP (DIG-labeled) in the presence of T3 RNA polymerase and incubated at 37° C for 6 hours. Adding DNaseI to the reaction mixture, it was incubated for 10 minutes to degrade the template DNA. After adding a 1/10 volume of 5 M sodium acetate and an equal volume of isopropanol were added to this reaction solution, it was centrifuged at 15,000 g x 15 min to recover the synthesized RNA as a precipitate. The precipitate was dissolved in RNase-free sterile distilled water.

[0188]

Fourthly, for IL-10, pH15C, a plasmid DNA containing human IL-10 cDNA was extracted and purified using Plasmid Midi Kit (QIAGEN) from 50 ml culture of an *E. coli* strain (ATCC#104607) carrying the plasmid.

The plasmid was digested with restriction enzyme BamHI, and the isolated IL-10 cDNA fragment was linked to the BamHI restriction site of a pBluescript KS(+) vector for RNA synthesis using a DNA Ligation kit version 2 (Takara). The recombinant plasmid was introduced into competent cells of *E. coli* JM109 (Takara). The recombinant plasmid DNA was extracted and purified from 50 ml culture of the resulting *E. coli* transformants using a Plasmid Midi Kit (QIAGEN).

[0189]

The recombinant plasmid (phIL-10#10) was digested with restriction enzyme SmaI. After treating the linearized DNA with phenol/chloroform, it was purified by ethanol precipitation to prepare a template for RNA probe synthesis. This template DNA (5 μg) was mixed with 1.1 mM ATP, 0.9 mM CTP, 0.9 mM GTP, 0.6 mM UTP and 0.50 mM UTP (DIG-labeled) in the presence of T7 RNA polymerase and incubated at 37° C for 6 hours. Adding DNaseI to the reaction mixture, it was incubated for 10 minutes to degrade the template DNA. After adding a 1/10 volume of 5 M sodium acetate and an equal volume of isopropanol to this reaction solution, it was centrifuged at 15,000 g x 15 min to recover the synthesized RNA as a precipitate. The precipitate was dissolved in RNase-free sterile distilled water.

[0190]

The full-length RNA probes for γ-IF, TNF-β, IL-5 and IL-10 obtained above were highly fragmented in the same manner as those for IL-2 and IL-4. 10 μg of each RNA probe was dissolved in 10 μl of the above-mentioned alkali-denaturing solution and incubated at 60° C for 10-15 minutes. Adding 10 μl of 3 M sodium acetate and 350 μl of ethanol

to the denaturing solution, it was cooled at -20° C for 30 minutes and centrifuged at 16 krpm for 20 minutes to precipitate the RNA probe. The precipitate was rinsed with 70% ethanol, dried, and dissolved in 50 μl of RNase-free sterile distilled water to prepare an alkaline denatured γ-IF, TNF- β, IL-5 or IL-10 RNA probe. Washing the cells fixed on the bottom of the chamber 3 times with PBS(-), the cells were treated with a 0.1% Triton X-100/PBS solution at room temperature for 5 minutes to permeabilize the cells. Washing 3 times with PBS(-), the permeabilized cells were treated with 0.2 N HCl at room temperature for 10 minutes.

[0191]

After washing the cells with PBS(-), it was incubated for 5 minutes at 37° C with 1 μg/ml Proteinase K in PBS(-). Washing the resulting cells with PBS(-), it was fixed again with 4% paraformaldehyde/PBS (pH 7.4) for 30 minutes. Washing the fixed cells twice with 2 mg/ml glycine in PBS for 15 minutes each, they were treated with 50% deionized formaldehyde/2 x SSC solution (hereunder, Soln. A) for 30 minutes to prepare a hybridization solution (50% deionized formaldehyde, 5 x Denhardt, 2 x SSC, alkaline denatured IL-2, γ-IF, TNF- β, IL-4, IL-5, or IL-10 RNA probe (1 μg/ml)). Denaturing the solution at 90° C for 10 minutes, it was cooled on ice. Adding 100 μl of the same solution to the cells, they were incubated overnight at 45° C.

[0192]

After the hybridization, the cells were washed with Soln. A for 5 minutes at 45° C, and then washed twice with Soln. B (10 mM Tris·HCl (pH 8.5), 500 mM NaCl) for 5 minutes each. The cells were treated with 20 mg/ml RNase A/Soln. B (pretreated at 90° C for 10 minutes) at 37° C

for 20 minutes. After washing the cells with Soln. A and Soln. C (50% deionized formaldehyde/1 x SSC) for 30 minutes each at 45° C, they were washed with Soln. C at room temperature for 20 minutes. Washing the cells with Buffer 1 (100 mM maleic acid, 150 mM NaCl (pH 7.5)) (2 x 5 min), they were treated with Buffer 2 (1% Blocking Reagent (Boehringer Mannheim) in Buffer 1) at room temperature for 20 minutes. After washing the cells twice with Buffer 1, FITC-labeled anti-DIG antibody (Fab, diluted with Buffer 2 by 100 times, protein concentration: approximately 1 μ g/ml) was added to the cells and they were incubated for at least 30 minutes. Washing the cells 3 times with PBS(-), they were observed under a fluorescence microscope to examine the ratio of IL-2, γ-IF, TNF β, IL-4, IL-5 or IL-10 mRNA carrying cells (TH1 or TH2) among the cells in the entire visual field. The cell type (TH1 or TH2) was determined for the selectively separated cells by a cell sorting function.

[0193]

The cells selectively separated by the cell sorter in (19) and (24) were positive for IL-2, γ-IF and TNF-β mRNA and negative for IL-4, IL-5 and IL-10 (Figs. 87 and 91), indicating that all the cells examined were TH1-specific cytokine-producing cells. On the other hand, the cells selectively separated by the cell sorter in (21) and (27) were negative for IL-2, γ-IF and TNF-β mRNA and positive for IL-4, IL-5 and IL-10 (Figs. 89 and 93), identifying all the cells examined as TH2-specific cytokine-producing cells.

[0194]

<u>(30) Selective separation of live TH1 and TH2 cells expressing specific genes based on difference in fluorescence intensity</u>

All the selective separation experiments of TH1 or TH2 cells based on the fluorescently labeled IL-2 or IL-4 mRNA were summarized in Table 9. In this table, their mRNAs were utilized as markers to isolate the objective TH cells throughout the experimental results from (18), (20), (23) and (26).

[0195]

Table 9

Mixing Ratios			Flow Cytometry			
			Before		After	
No.	Marker	Target cell type	FRET (D/A) positive cells (%)	IL-2 or IL-4 mRNA carrying cells (%)	FRET (D/A) positive cells (%)	IL-2 or IL-4 mRNA carrying cells (%)

(18)	IL-2 mRNA	TH-1	12.0	20.8	100	100
(20)	IL-4 mRNA	TH-2	4.9	7.1	100	100
(23)	IL-2 mRNA	TH-1	4.3	7.9	100	100
(26)	IL-4 mRNA	TH-2	2.4	2.2	100	100

[0196]

In (18), the IL-2 expressing cells (TH1) which presented only at 12% (Fig. 60, live cell fluorescent observation results) or 20% (Fig. 86, FISH experiment results) were concentrated to 100% by the separation method utilizing the difference in fluorescence intensities as described in (19). The IL-4 expressing cells (TH2) presented only at 4.9% (Fig. 68, fluorescent observation results) or 7.1% (Fig. 88, FISH experiment results) before flow cytometry as described in (20). However, all the cells obtained by the selective separation method of (21) were IL-4 mRNA carrying cells.

[0197]

Furthermore, the ratio of TH1 to TH2 cells was artificially shifted toward TH2 to mimic immune diseases with overactivation of TH2 cells in (23). Compared with (18), TH1 was notably reduced to 4.3% (Fig. 74, fluorescent observation results) or 7.9% (Fig. 90, FISH experiment results). However, TH1 cells were obtained at 100% purity by the selective separation method as shown in (24). In contrast to (23), the balance between TH1 and TH2 was artificially shifted toward TH1 in (26) to mimic immune diseases with overwhelming presence of TH1. Compared with (25), TH2 cells were reduced to 2.4% (Fig. 80, fluorescent observation

results) or 2.3% (Fig. 92, FISH experiment results). However, TH2 cells were obtained at 100% purity by the selective separation method as shown in (27).

[0198]

To confirm the above-mentioned results, mRNA of TH1- or TH2-specific cytokine was detected in (29). It was demonstrated that all the cells separated by the cell sorter in (19) and (24) from the cells of (18) and (23) were TH1, while all the cells selectively separated in (21) and (27) from the cells of (20) and (26) were TH2.

[0199]

Throughout all these results, it was concluded that TH1 or TH2 cells are selectively separated with complete selectivity (100%) from cells containing the both cell types by the separation method utilizing mRNA of IL-2 or IL-4, a specific cytokine for TH1 or TH2 cells, respectively.

[0200]

[Effect of the Invention]

As described above, according to the present invention, it is possible to provide a separation method which allows one to selectively separate and obtain the objective cells, that is, the cells which have expressed a specific gene, when there are no cell surface molecules usable as markers in the cell, or when the cell surface molecules cannot be distinguished from each other even if they are present in the cell, or even when the molecules to be the markers are liberated into the extracellular fluid.

SEQUENCE LISTING

<110> Laboratory of Molecular Biophotonics

<120> Method for selectively separating live cells that have expressed a specific gene

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[BRIEF DESCRIPTION OF DRAWINGS]

[FIG. 1]

Fig. 1 is a listing of the entire base sequence of IL-2 mRNA and the base sequences of oligo DNA probes.

[FIG. 2]

Fig. 2 is a listing of the entire base sequence of IL-4 mRNA and the base sequences of oligo DNA probes.

[FIG. 3]

Fig. 3 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe 228-242(D) and the acceptor probe 243-257(A)being adjacently hybridized to IL-2 RNA.

[FIG. 4]

Fig. 4 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-2 198-212(D) and the acceptor probe IL-2 213-227(A) being adjacently hybridized to IL-2 RNA.

[FIG. 5]

Fig. 5 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-2 77-91(D) and the acceptor probe IL-2 92-106(A) being adjacently hybridized to IL-2 RNA.

[FIG. 6]

Fig. 6 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-2 287-301(D) and the acceptor probe IL-2 302-316(A) be adjacently hybridized to IL-2 RNA.

[FIG. 7]

Fig. 7 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-2 342-356(D) and the acceptor probe IL-2 357-371(A) being adjacently hybridized to IL-2 RNA.

[FIG. 8]

Fig. 8 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-4 70-84(D) and the acceptor probe IL-4 85-99(A) being adjacently hybridized to IL-4 RNA.

[FIG. 9]

Fig. 9 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-4 119-133(D) and the acceptor probe IL-4 134-148(A) being adjacently hybridized to IL-4 RNA.

[FIG. 10]

Fig. 10 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-4 176-190(D) and the acceptor probe IL-4 191-205(A) being adjacently hybridized to IL-4 RNA.

[FIG. 11]

Fig. 11 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-4 265-279(D) and the acceptor probe IL-4 280-294(A) being adjacently hybridized to IL-4 RNA.

[FIG. 12]

Fig. 12 is a graph of a fluorescence spectrum of a hybrid formed by the donor probe IL-4 376-390(D) and the acceptor probe IL-4 391-405(A) adjacently hybridized to IL-4 RNA.

[FIG. 13]

Fig. 13 is a chromatogram of HPLC obtained when a mixture solution of the donor probe IL-2 342-356(D) and IL-2 RNA was separated by HPLC.

[FIG. 14]

Fig. 14 is a chromatogram of HPLC obtained when a mixture solution of the acceptor probe IL-2 357-371(D) and IL-2 RNA was separated by HPLC.

[FIG. 15]

Fig. 15 is a chromatogram of HPLC obtained when a mixture of the donor probe IL-4 119-133(D) and IL-4 RNA was separated by HPLC.

[FIG. 16]

Fig. 16 is a chromatogram of HPLC obtained when a mixture of the acceptor probe IL-4 134-148(D) and IL-4 RNA was separated by HPLC.

[FIG. 17]

Fig. 17 is a chromatogram of HPLC obtained when a mixture of the donor probe IL-4 265-279(D) and IL-4 RNA was separated by HPLC.

[FIG. 18]

Fig. 18 is a chromatogram of HPLC obtained when a mixture of the acceptor probe IL-4 280-294(D) and IL-4 RNA was separated by HPLC.

[FIG. 19]

Fig. 19 is a graph showing the amount of IL-2 secreted by Jurkat E6-1 cells associated with the expression induction treatment of IL-2, fluorescence micrographs obtained when IL-2 mRNA in the cell extract was fluorescently detected, and the number of molecules of the intracellular IL-2 mRNA.

[FIG. 20]

Fig. 20 is a set of fluorescence micrographs obtained for those to which DIG-labeled dUTP and reverse transcriptase were added after oligonucleotides (oligo dT or oligo dA) had been introduced to IL-2 expression-induced cells or IL-2 expression-uninduced cells in the fixed state, those to which the oligonucleotides were not introduced thereafter, and those to which neither of them was introduced nor was added.

[FIG. 21]

Fig. 21 is a set of fluorescence micrographs obtained when hybrids were formed between IL-2 mRNA in the IL-2 expression-induced cells or IL-2 expression-uninduced cells in the fixed state and various probes (non-fluorescent markers), and the hybrids were fluorescently detected.

[FIG. 22]

Fig. 22 is a graph showing the results obtained when hybrids were formed between IL-2 mRNA in the IL-2 expression-induced cells or IL-2 expression-uninduced cells in the fixed state and various probes (non-fluorescent markers) were fluorescently detected, and the fluorescence intensities were normalized based on the values of fluorescence intensity emitted from the fluorescent labeled compounds formed between total mRNA in said cells and oligo dT.

[FIG. 23]

Fig. 23 is a set of fluorescence micrographs showing D/A, D/D and A/A images of hybrids formed by the three components, IL-2 mRNA in IL-2 expression-induced cells or IL-2 expression-uninduced cells in the fixed state, respective donor probes and respective acceptor probes upon excitation of the donor fluorescent dyes of the hybrids.

[FIG. 24]

Fig. 24 is a graph showing the results obtained when the fluorescence of the acceptor fluorescence dyes was measured upon excitation of the donor fluorescent dyes of the hybrids formed by the three molecules, IL-2 mRNA in IL-2 expression-induced cells or IL-2 expression-uninduced cells in the fixed state, respective donor probes and respective acceptor probes, and the measured fluorescence was standardized based the measured values of fluorescence of the acceptor fluorescent dyes

upon excitation of the acceptor fluorescent dyes representing all the acceptor probes in said cells.

[FIG. 25]

Fig. 25 is a set of fluorescence micrographs of D/A, D/D and A/A images of a hybrid formed by the three molecules, IL-2 mRNA in IL-2 expression-induced cells or IL-2 expression-uninduced cells in the living state, IL-2 342-356(D) and IL-2 357-371(A), and the corresponding phase contrast micrograph.

[FIG. 26]

Fig. 26 is a dot plot of the results based on forward scattering light and side scattering light for a cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 100:0 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 27]

Fig. 27 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 100:0 when subjected to flow cytometry (R2 is the region selected for fluorescing cells due to FRET).

[FIG. 28]

Fig. 28 is a dot plot of the results based on forward scattering light and side scattering light for a cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 0:100 when subjected to flow cytometry.

[FIG. 29]

Fig. 29 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 0:100 when subjected to flow cytometry.

[FIG. 30]

Fig. 30 is a dot plot of the results based on forward scattering light and side scattering light for a cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 50:50 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 31]

Fig. 31 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 50:50 when subjected to flow cytometry (R2 is the region selected for fluorescing cells due to FRET).

[FIG. 32]

Fig. 32 is a dot plot of the results based on forward scattering light and side scattering light for a cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 20:80 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 33]

Fig. 33 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 20:80 when subjected to flow cytometry (R2 is the region selected for fluorescing cells due to FRET).

[FIG. 34]

Fig. 34 is a dot plot of the results based on forward scattering light and side scattering light for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells 100:0 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of Fig. 26 and the R2 gate of Fig. 27, and the resulting cell group was again subjected to flow cytometry.

[FIG. 35]

Fig. 35 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 100:0 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of Fig. 26 and the R2 gate of Fig. 27, and the resulting cell group was again subjected to flow cytometry.

[FIG. 36]

Fig. 36 is a dot plot of the results based on forward scattering light and side scattering light for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was

50:50 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of Fig. 30 and the R2 gate of Fig. 31, and the resulting cell group was again subjected to flow cytometry.

[FIG. 37]

Fig. 37 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 50:50 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of Fig. 30 and the R2 gate of Fig. 31, and the resulting cell group was again subjected to flow cytometry.

[FIG. 38]

Fig. 38 is a dot plot of the results based on forward scattering light and side scattering light for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 20:80 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of Fig. 32 and the R2 gate of Fig. 33, and the resulting cell group was again subjected to flow cytometry.

[FIG. 39]

Fig. 39 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET for the cell group where the mixing ratio of the IL-2 expression-induced cells to the IL-2 expression-uninduced cells was 20:80 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of

Fig. 32 and the R2 gate of Fig. 33, and the resulting cell group was again subjected to flow cytometry.

[FIG. 40]

Fig. 40 is a set of micrographs showing D/A, D/D and A/A images, and the corresponding phase contrast micrograph of the cell group where the mixing ratio of the IL-2 expression-induced cells to the expression-uninduced cells in the live state was 100:0 before subjected to flow cytometry.

[FIG. 41]

Fig. 41 is a set of micrographs showing D/A, D/D and A/A images, and the corresponding phase contrast micrograph of the cell group where the mixing ratio of the IL-2 expression-induced cells to the expression-uninduced cells in the live state was 100:0 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of Fig. 26 and the R2 gate of Fig. 27.

[FIG. 42]

Fig. 42 is a set of micrographs showing D/A, D/D and A/A images, and the corresponding phase contrast micrograph of the cell group where the mixing ratio of the IL-2 expression-induced cells to the expression-uninduced cells in the live state was 50:50 before subjected to flow cytometry.

[FIG. 43]

Fig. 43 is a set of micrographs showing D/A, D/D and A/A images, and the corresponding phase contrast micrograph of the cell group where the mixing ratio of the IL-2 expression-induced cells to the expression-uninduced cells in the live state was 50:50 when the selective separation

was conducted according to flow cytometry by gating with the R1 gate of Fig. 40 and the R2 gate of Fig. 31.

[FIG. 44]

Fig. 44 is a set of micrographs showing D/A, D/D and A/A images, and the corresponding phase contrast micrograph of the cell group where the mixing ratio of the IL-2 expression-induced cells to the expression-uninduced cells in the live state was 20:80 before subjected to flow cytometry.

[FIG. 45]

Fig. 45 is a set of micrographs showing D/A, D/D and A/A images, and the corresponding phase contrast micrograph of the cell group where the mixing ratio of the IL-2 expression-induced cells to the expression-uninduced cells in the live state was 20:80 when the selective separation was conducted according to flow cytometry by gating with the R1 gate of Fig. 32 and the R2 gate of Fig. 33.

[FIG. 46]

Fig. 46 is a set of micrographs showing D/A, D/D and A/A images, and the corresponding phase contrast micrograph of the cell group where the mixing ratio of the IL-2 expression-induced cells to the expression-uninduced cells in the live state was 0:100 before subjected to flow cytometry.

[FIG. 47]

Fig. 47 is a fluorescence micrograph obtained when the cell group obtained by mixing IL-2 expression-induced cells and IL-2 expression-uninduced cells at the ratio of 100:0 before flow cytometry thereof was fixed to a glass-bottomed dish, hybrids were formed between the cellular

IL-2 mRNA in the fixed state and RNA probe for IL-2 RNA s, and the hybrids were fluorescently detected.

[FIG. 48]

Fig. 48 is a fluorescence micrograph obtained when the cell group obtained by mixing IL-2 expression-induced cells and IL-2 expression-uninduced cells at the ratio of 100:0 was subjected to selective separation according to flow cytometry by gating with the R1 gate of Fig. 26 and the R2 gate of Fig. 27, the resulting cell group was fixed to a glass-bottomed dish, hybrids were formed between the cellular IL-2 mRNA in the fixed state and RNA probe for IL-2 RNA s, and the hybrids were fluorescently detected.

[FIG. 49]

Fig. 49 is a fluorescence micrograph obtained when the cell group obtained by mixing IL-2 expression-induced cells and IL-2 expression-uninduced cells at the ratio of 0:100 before flow cytometry thereof was fixed to a glass-bottomed dish, hybrids were formed between the cellular IL-2 mRNA in the fixed state and RNA probe for IL-2 RNA s, and the hybrids were fluorescently detected.

[FIG. 50]

Fig. 50 is a fluorescence micrograph obtained when the cell group obtained by mixing IL-2 expression-induced cells and IL-2 expression-uninduced cells at the ratio of 50:50 before flow cytometry thereof was fixed to a glass-bottomed dish, hybrids were formed between the cellular IL-2 mRNA in the fixed state and RNA probe for IL-2 RNA s, and the hybrids were fluorescently detected.

[FIG. 51]

Fig. 51 is a fluorescence micrograph obtained when the cell group obtained by mixing IL-2 expression-induced cells and IL-2 expression-uninduced cells at the ratio of 50:50 was subjected to selective separation according to flow cytometry by gating with the R1 gate of Fig. 30 and the R2 gate of Fig. 31, the resulting cell group was fixed to a glass-bottomed dish, hybrids were formed between the cellular IL-2 mRNA in the fixed state and RNA probe for IL-2 RNA s, and the hybrids were fluorescently detected.

[FIG. 52]

Fig. 52 is a fluorescence micrograph obtained when the cell group obtained by mixing IL-2 expression-induced cells and IL-2 expression-uninduced cells at the ratio of 20:80 before flow cytometry thereof was fixed to a glass-bottomed dish, hybrids were formed between the cellular IL-2 mRNA in the fixed state and RNA probe for IL-2 RNA s, and the hybrids were fluorescently detected.

[FIG. 53]

Fig. 53 is a fluorescence micrograph obtained when the cell group obtained by mixing IL-2 expression-induced cells and IL-2 expression-uninduced cells at the ratio of 20:80 was subjected to selective separation according to flow cytometry by gating with the R1 gate of Fig. 32 and the R2 gate of Fig. 33, the resulting cell group was fixed to a glass-bottomed dish, hybrids were then formed between the cellular IL-2 mRNA in the fixed state and RNA probe for IL-2 RNA s, and the hybrids were fluorescently detected.

[FIG. 54]

Fig. 54 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for lymphocytes separated from human peripheral blood when subjected to flow cytometry.

[FIG. 55]

Fig. 55 is a dot plot of the results of lymphocytes separated from human peripheral blood that were fluorescently labeled on cell surfaces thereof with a control antibody when subjected to flow cytometry.

[FIG. 56]

Fig. 56 is a dot plot of the results of lymphocytes separated from human peripheral blood that were fluorescently labeled at CD4 (CD4 FITC) and CD8 (CD8 PE) on cell surfaces thereof when subjected to flow cytometry.

[FIG. 57]

Fig. 57 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for CD4+ cells separated from human peripheral blood with a CD4+ cell separating column when subjected to flow cytometry.

[FIG. 58]

Fig. 58 is a dot plot of the results of CD4+ cells separated from human peripheral blood with a CD4+ cell separating column that were fluorescently labeled with a control antibody when subjected to flow cytometry.

[FIG. 59]

Fig. 59 is a dot plot of the results of CD4+ cells separated from human peripheral blood with a CD4+ cell separating column that were

fluorescently labeled at CD4 (CD4 FITC) and CD8 (CD8 PE) on cell surfaces thereof when subjected to flow cytometry.

[FIG. 60]

Fig. 60 is a set of micrographs showing A/A, D/A and D/D fluorescence images of a hybrid formed by the three components, intracellular IL-2 mRNA of a CD4+ cell (helper T cell) in the live state, IL-2 342-356(D) and IL-2 357-371(A), and the corresponding phase contrast micrograph.

[FIG. 61]

Fig. 61 is a set of micrographs showing A/A, D/A and D/D fluorescence images, and the corresponding phase contrast micrograph, of a cell group obtained by selective separation of the CD4+ cell group of Fig. 60 in the live state according to flow cytometry by gating with the R1 gate of Fig. 62 and the R2 gate of Fig. 63.

[FIG. 62]

Fig. 62 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell group of CD4+ cells (helper T cells) of Fig. 60 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 63]

Fig. 63 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, of the cell group of CD4+ cells (helper T cells) of Fig. 60 when subjected to flow cytometry (R2 is the region selected for fluorescing cells due to FRET).

[FIG. 64]

Fig. 64 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell sorter-separated cell group of Fig. 61 when subjected to flow cytometry (where R1 is the region selected for live cells to be measured).

[FIG. 65]

Fig. 65 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, for the cell sorter-separated cell group of Fig. 61 when subjected to flow cytometry (R2 is the region selected for fluorescing cells based on FRET).

[FIG. 66]

Fig. 66 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for a cell group of CD4+ cells (helper T cells) with no fluorescent probes introduced, when subjected to flow cytometry.

[FIG. 67]

Fig. 67 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, for the cell group of CD4+ cells (helper T cells) with no fluorescent probes introduced, when subjected to flow cytometry.

[FIG. 68]

Fig. 68 is a set of micrographs showing A/A, D/A and D/D fluorescence images of a hybrid formed by the three components, intracellular IL-4 mRNA of CD4+ cells (helper T cells) in the live state, IL-4

265-279(D) and IL-4 280-294(A) and the corresponding phase contrast micrograph.

[FIG. 69]

Fig. 69 is a set of micrographs showing A/A, D/A and D/D fluorescence images, and the corresponding phase contrast micrograph, of the cell group obtained by selective separation of CD4+ cell group in the live state of Fig. 68 according to flow cytometry by gating with the R1 gate of Fig. 70 and the R2 gate of Fig. 71.

[FIG. 70]

Fig. 70 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell group of CD4+ cells (helper T cells) in the live state of Fig. 68 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 71]

Fig. 71 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, for the cell group of CD4+ cells (helper T cells) of Fig. 68 when subjected to flow cytometry (R2 is selected for fluorescing cells due to FRET).

[FIG. 72]

Fig. 72 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell sorter-separated cell group of Fig. 69 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 73]

Fig. 73 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, for the cell sorter-separated cell group of Fig. 35 when subjected to flow cytometry (R2 is the region selected for fluorescing cells based on FRET).

[FIG. 74]

Fig. 74 is a set of micrographs showing A/A, D/A and D/D fluorescence images of a hybrid formed by the three components, intracellular IL-2 mRNA of TH2-induced CD4+ cell in the live state (helper T cell), IL-2 342-356(D) and IL-2 357-371(A) and the corresponding phase contrast micrograph.

[FIG. 75]

Fig. 75 is a set of micrographs showing A/A, D/A and D/D fluorescence images, and the corresponding phase contrast micrograph, of the cell group obtained by selective separation of CD4+ cell group in the live state of Fig. 74 according to flow cytometry by gating with the R1 gate of Fig. 76 and the R2 gate of Fig. 77.

[FIG. 76]

Fig. 76 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell group of CD4+ cells (helper T cells) of Fig. 74 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 77]

Fig. 77 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, for the cell

group of CD4+ cells (helper T cells) of Fig. 74 when subjected to flow cytometry (R2 is the region selected for fluorescing cells based on FRET).

[FIG. 78]

Fig. 78 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell sorter-separated cell group of Fig. 75 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 79]

Fig. 79 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, for the cell sorter-separated cell group of Fig. 75 when subjected to flow cytometry (R2 is the region selected for fluorescing cells based on FRET).

[FIG. 80]

Fig. 80 is a set of micrographs showing A/A, D/A and D/D fluorescence images of a hybrid formed by the three components, the intracellular IL-4 mRNA of CD4+ cells T1 induction-treated in the live state (helper T cells), IL-4 265-279(D) and IL-4 280-294(A) and the corresponding phase contrast micrograph.

[FIG. 81]

Fig. 81 is a set of micrographs showing A/A, D/A and D/D fluorescence images, and the corresponding phase contrast micrograph, of a cell group obtained by selective separation of CD4+ cell group of Fig. 80 in the live state according to flow cytometry by gating with the R1 gate of Fig. 82 and the R2 gate of Fig. 83.

[FIG. 82]

Fig. 82 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell group of CD4+ cells (helper T cells) of Fig. 80 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 83]

Fig. 83 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity of the energy acceptor fluorescent dye due to FRET, for the cell group of CD4+ cells (helper T cells) of Fig. 80 when subjected to flow cytometry (R2 is the region selected for fluorescing cells based on FRET).

[FIG. 84]

Fig. 84 is a dot plot of the results based on forward scattering light (FSC) and side scattering light (SSC) for the cell sorter-separated cell group of Fig. 81 when subjected to flow cytometry (R1 is the region selected for live cells to be measured).

[FIG. 85]

Fig. 85 is a dot plot of the results based on relative fluorescence intensity of the energy donor fluorescent dye and relative fluorescence intensity due of the energy acceptor fluorescent dye due to FRET, for the cell sorter-separated cell group of Fig. 81 when subjected to flow cytometry (R2 is the region selected for fluorescing cells based on FRET).

[FIG. 86]

Fig. 86 is a set of fluorescence micrographs obtained when the cell group of CD4+ cells (helper T cells) of Fig. 60 before flow cytometry thereof was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2 mRNA or IL-4 mRNA of the fixed cells and RNA

probe for IL-2 RNA s or IL-4 RNA probes, respectively, and the hybrids were fluorescently detected.

[FIG. 87]

Fig. 87 is a set of fluorescence micrographs obtained when the cell group of cells selectively separated with the cell sorter in Fig. 61 was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 mRNA of the fixed cells and IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 RNA probe, respectively, and the hybrids were fluorescently detected.

[FIG. 88]

Fig. 88 is a set of fluorescence micrographs obtained when the cell group of CD4+ cells (helper T cells) of Fig. 68 before flow cytometry thereof was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2 mRNA or IL-4 mRNA of the fixed cells and RNA probe for IL-2 RNA s or IL-4 RNA probes, respectively, and the hybrids were fluorescently detected.

[FIG. 89]

Fig. 89 is a set of fluorescence micrographs obtained when the cell group of cells selectively separated with the cell sorter in Fig. 69 was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 mRNA of the fixed cells and IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 RNA probes, respectively, and the hybrids were fluorescently detected.

[FIG. 90]

Fig. 90 is a set of fluorescence micrographs obtained when the cell group of CD4+ cells (helper T cells) of Fig. 74 before flow cytometry

thereof was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2 mRNA or IL-4 mRNA of the fixed cells and RNA probe for IL-2 RNA s or IL-4 RNA probes, and the hybrids were fluorescently detected.

[FIG. 91]

Fig. 91 is a set of fluorescence micrographs obtained when the cell group of cells selectively separated with the cell sorter in Fig. 75 was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 mRNA of the fixed cells and IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 RNA probes, respectively, and the hybrids were fluorescently detected.

[FIG. 92]

Fig. 92 is a set of fluorescence micrographs obtained when the cell group of CD4+ cells (helper T cells) of Fig. 80 before flow cytometry thereof was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2 mRNA or IL-4 mRNA of the fixed cells and RNA probe for IL-2 RNA s or IL-4 RNA probes, and the hybrids were fluorescently detected.

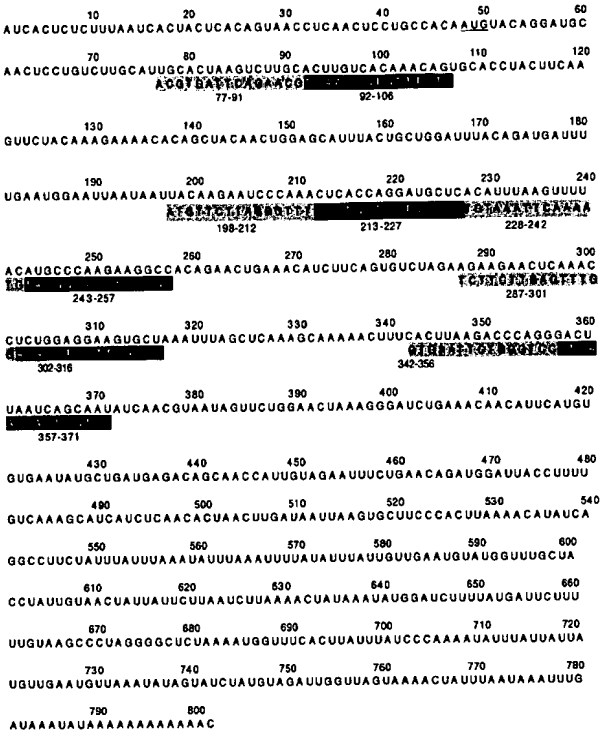
[FIG. 93]

Fig. 93 is a set of fluorescence micrographs obtained when the cell group of cells selectively separated with the cell sorter in Fig. 81 was fixed to the bottom of a cover glass chamber, hybrids were formed between the IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 mRNA of the fixed cells and IL-2, γ-IF, TNF-β, IL-4, IL-5 or IL-10 RNA probes, respectively, and the hybrids were fluorescently detected.

[FIG. 94]

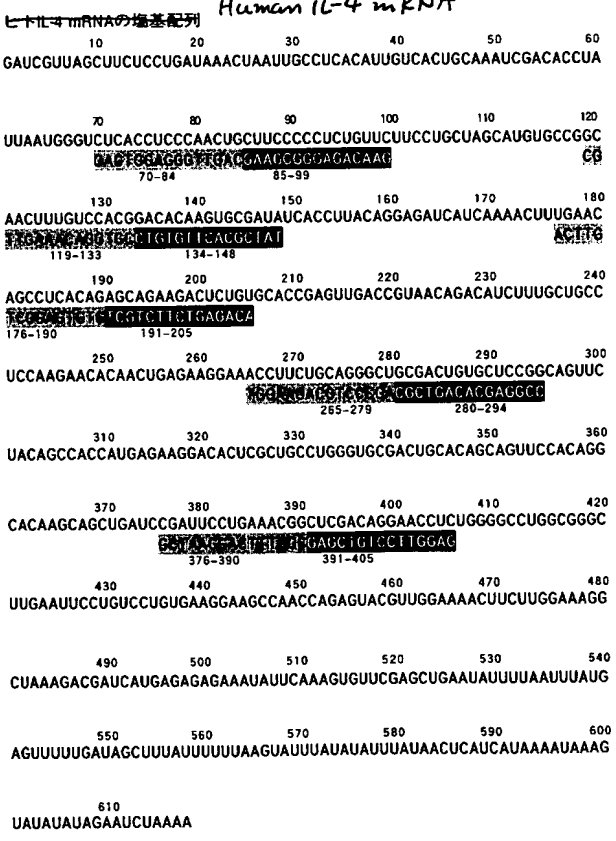
Fig. 94 is a representation showing the mutual relationship among different cells that constitute the immune system throughout their differentiation as well as the manner in which the different cells cooperate or restrain each other through cytokines such as interleukins to maintain homeostatis of immune functions of the living body.

【Fig.1】

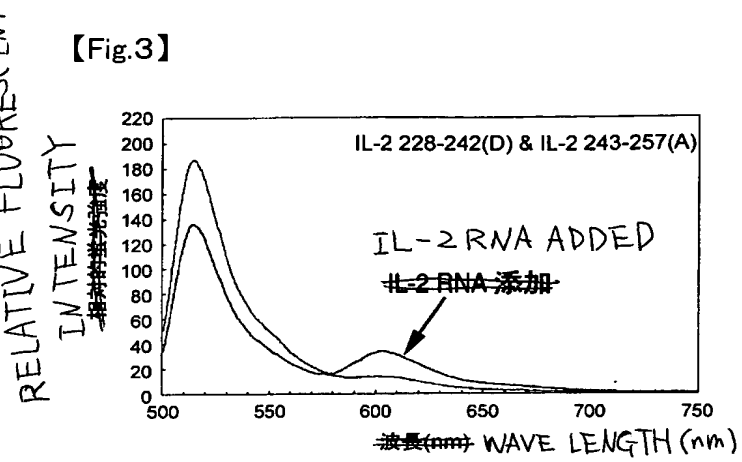


【Fig.2】

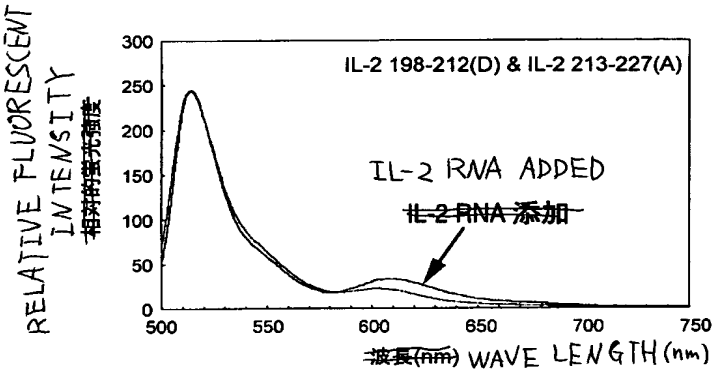
~~ヒトIL-4 mRNAの塩基配列~~ Human IL-4 mRNA



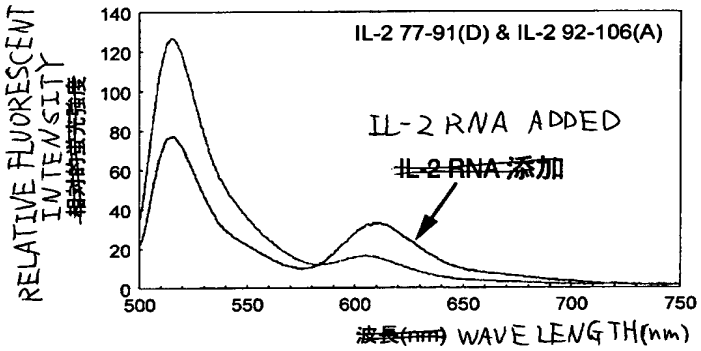
【Fig.3】



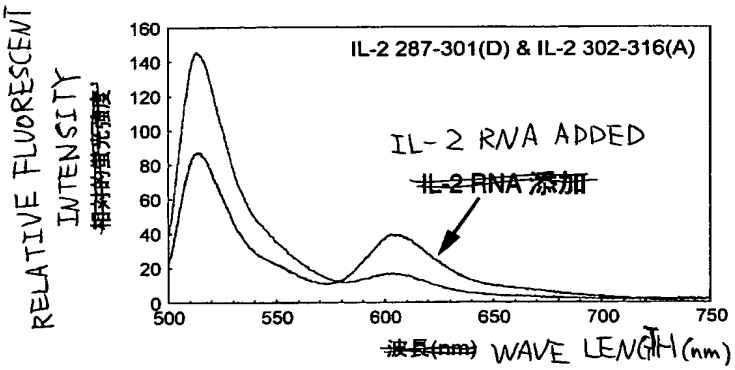
【Fig.4】



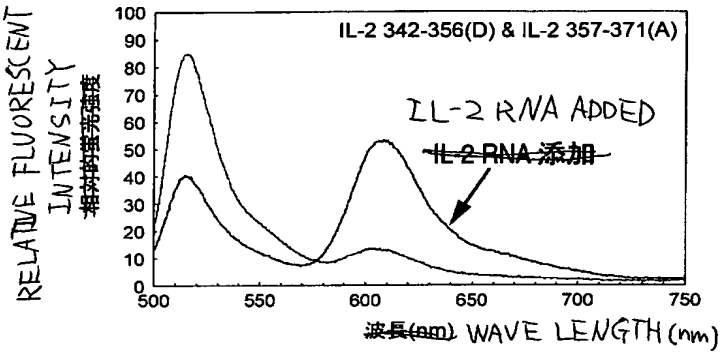
【Fig.5】



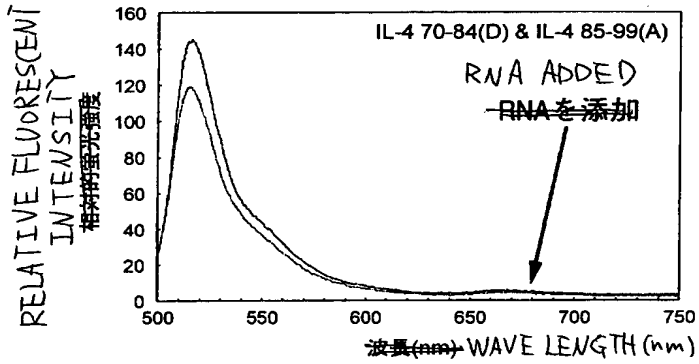
【Fig.6】



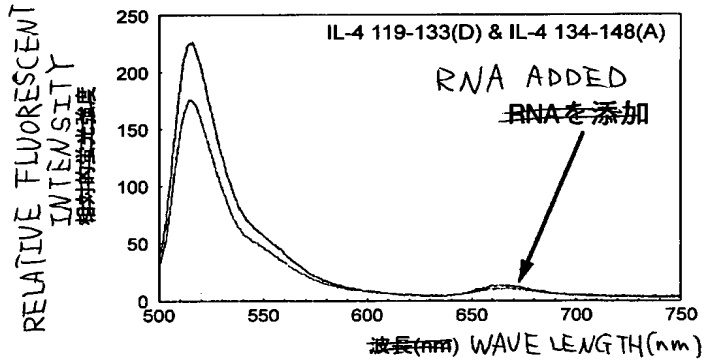
【Fig.7】



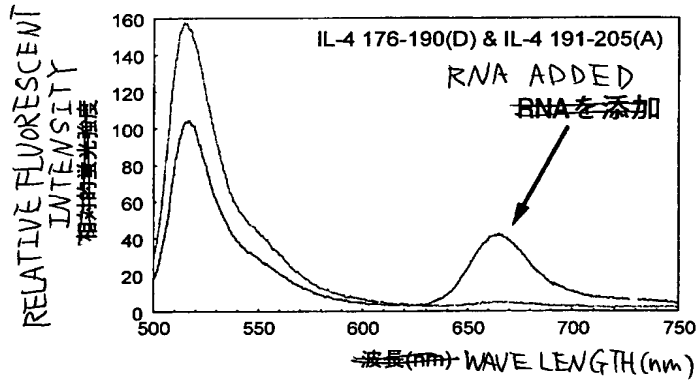
【Fig.8】



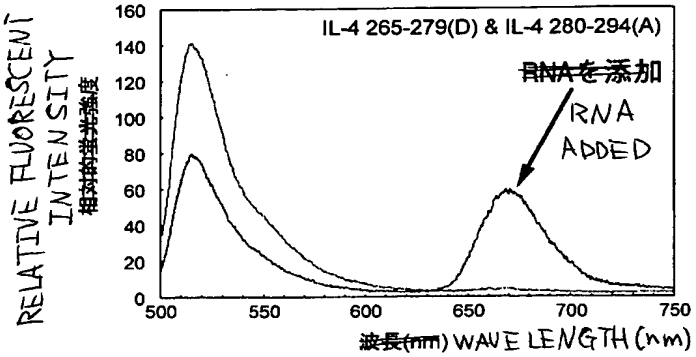
【Fig.9】



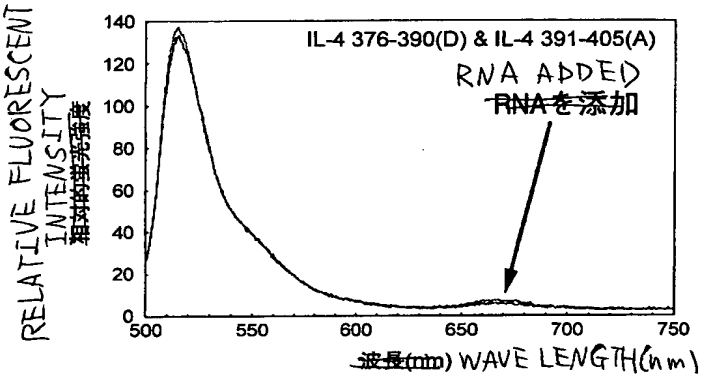
【Fig.10】



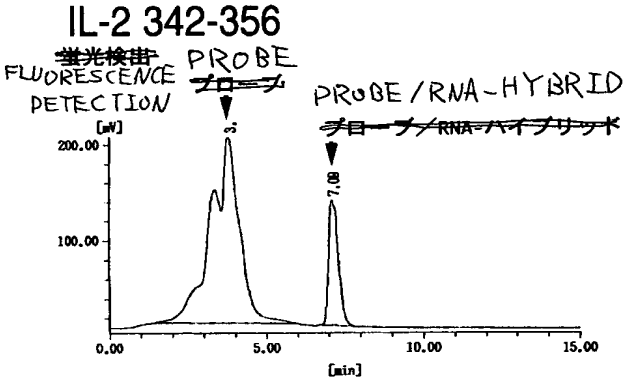
【Fig.11】



【Fig.12】



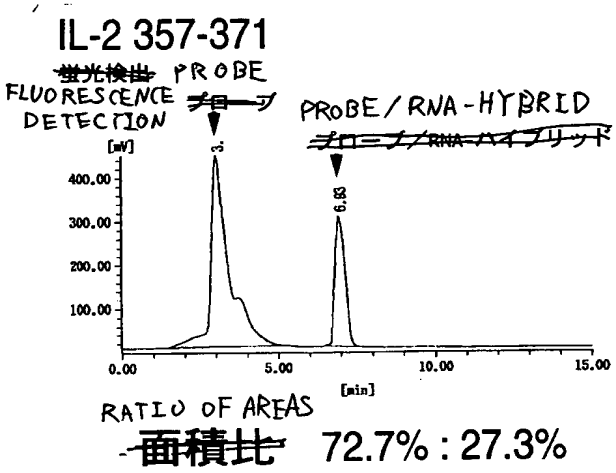
【Fig.13】



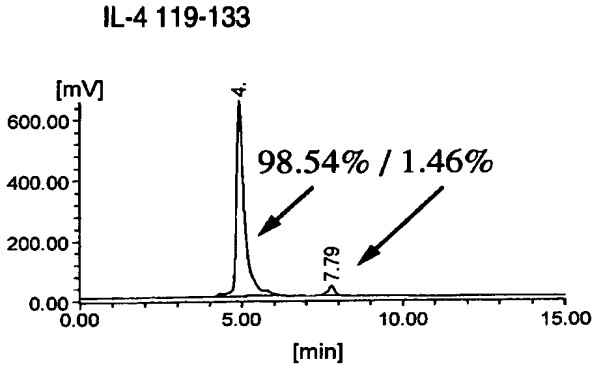
面積比 79.7% : 22.8%

RATIO OF AREAS

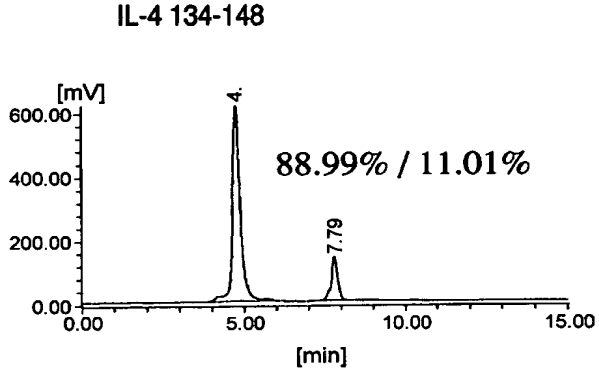
【Fig.14】



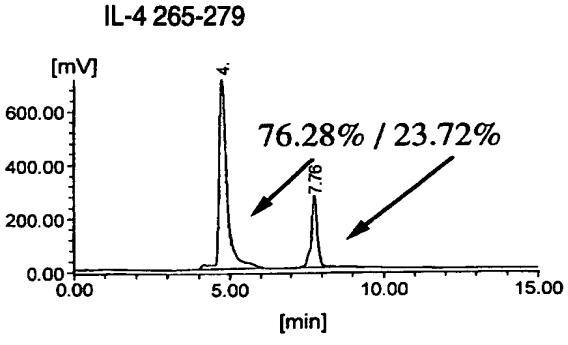
【Fig.15】



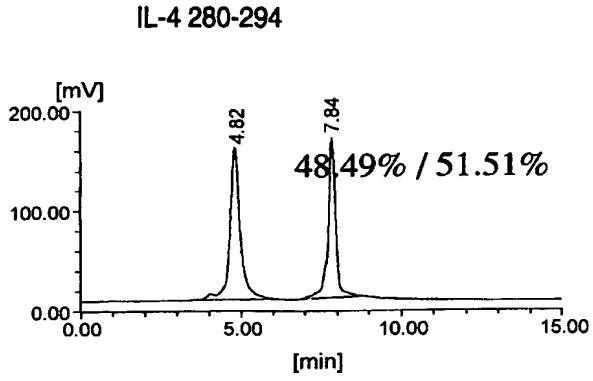
【Fig.16】



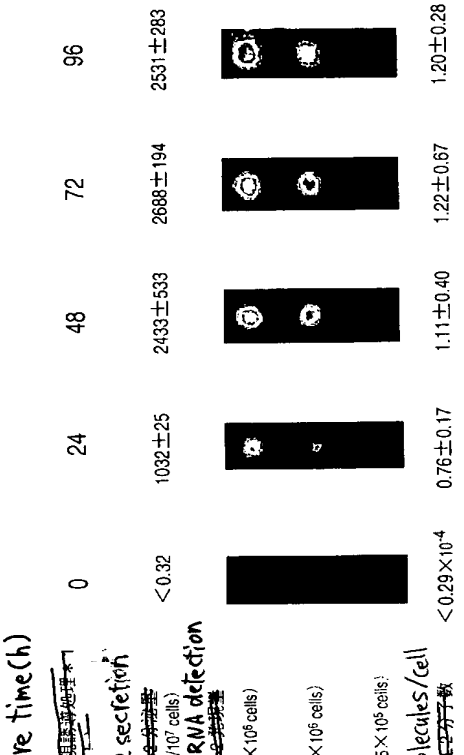
【Fig.17】



【Fig.18】



【Fig.19】

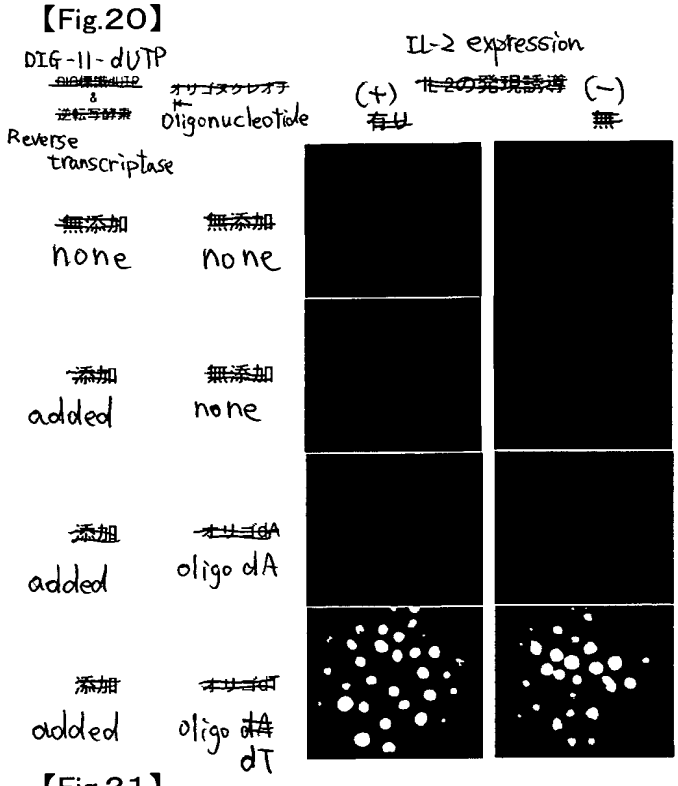


※1 Time after adding 10 nM PMA, 0.5 mg/ml anti-human CD3 antibody and anti-human CD28 antibody to cultured Jurkat E6-1 cells

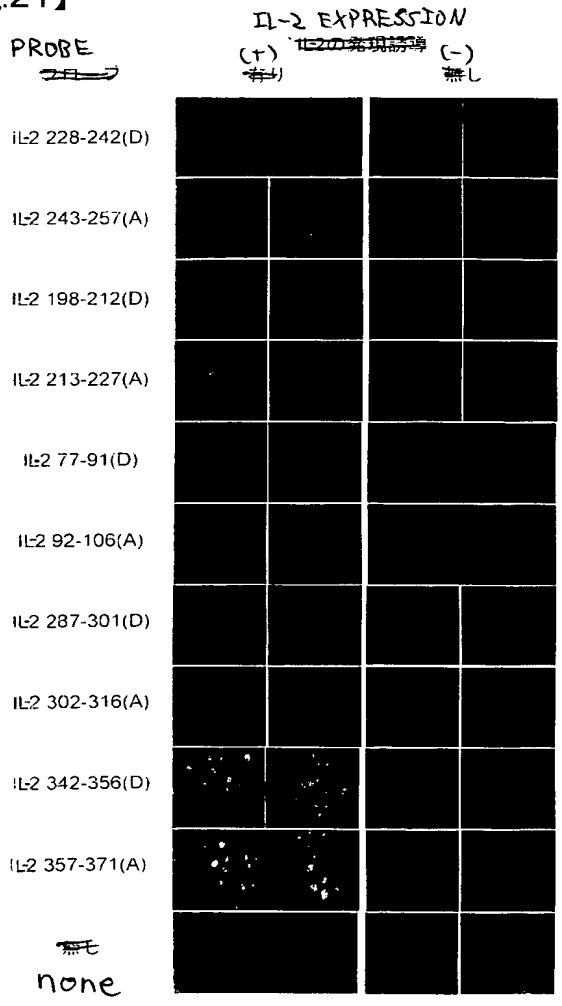
※2 The concentration of IL-2 in the culture supernatants measured by ELISA using anti-human IL-2 antibody

※3,4 Determination of cellular IL-2 expression level by dot blotting using RNA probe complementary to human IL-2 mRNA

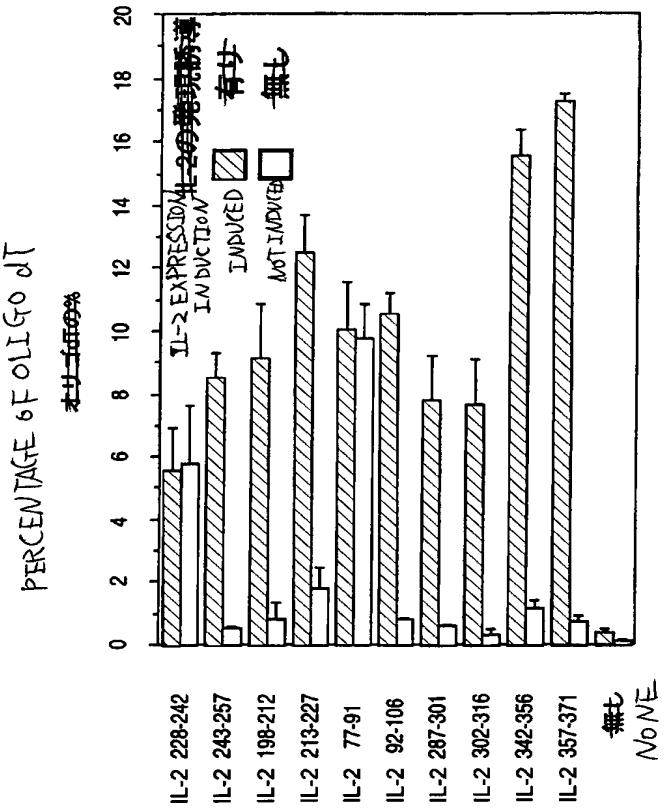
【Fig.20】


DIG-11-dUTP
~~DIG標識dUTP~~
&
~~逆転写酵素~~
Reverse transcriptase
~~オリゴヌクレオチド~~
Oligonucleotide
IL-2 expression
(+) ~~有り~~
~~IL-2の発現誘導~~
(-) ~~無~~
~~無添加~~ none
~~無添加~~ none
~~添加~~ added
~~無添加~~ none
~~添加~~ added
~~オリゴdA~~ oligo dA
~~添加~~ added
~~オリゴdT~~ oligo dT

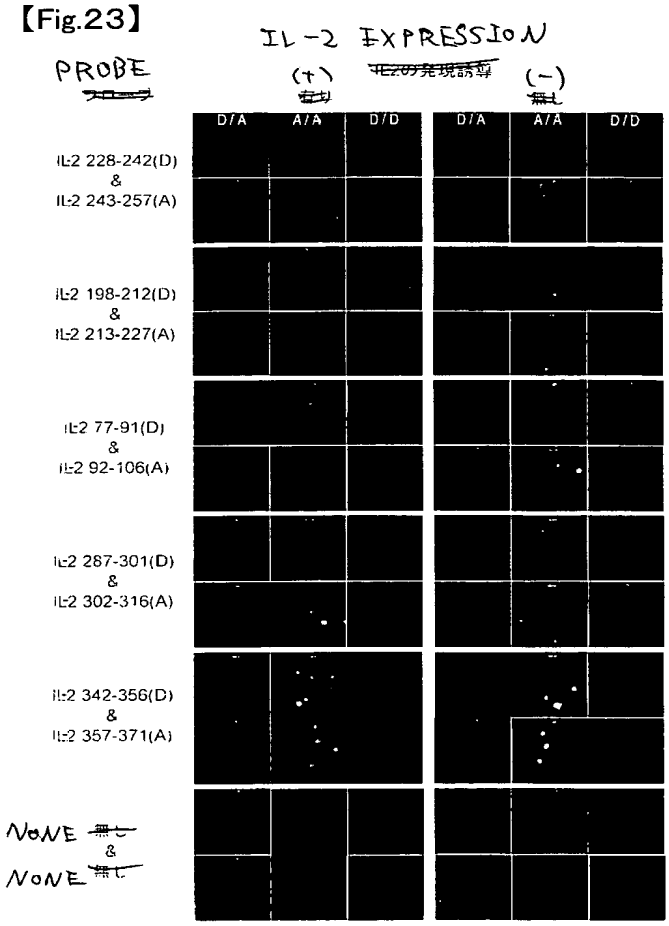
【Fig.21】


PROBE
~~プローブ~~
IL-2 EXPRESSION
(+) ~~有り~~
~~IL-2の発現誘導~~
(-) ~~無し~~
IL-2 228-242(D)
IL-2 243-257(A)
IL-2 198-212(D)
IL-2 213-227(A)
IL-2 77-91(D)
IL-2 92-106(A)
IL-2 287-301(D)
IL-2 302-316(A)
IL-2 342-356(D)
IL-2 357-371(A)
~~無し~~ none

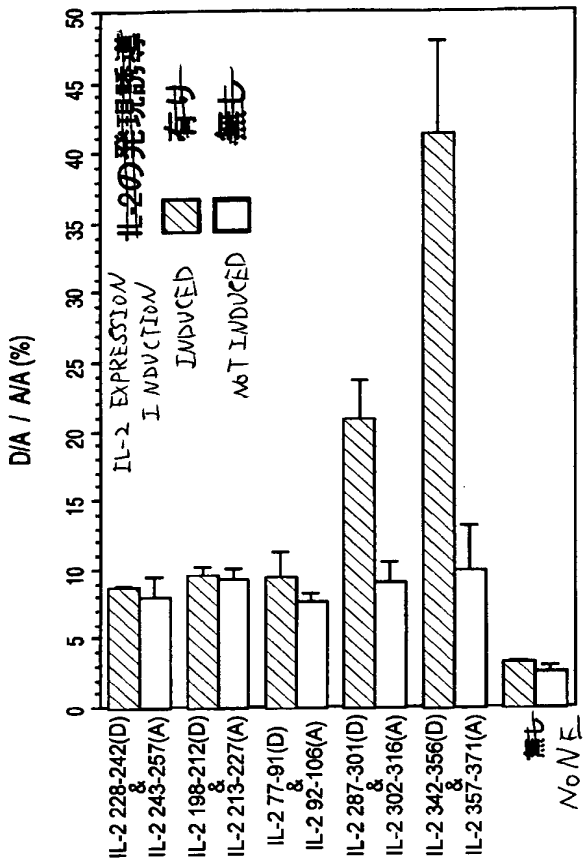
【Fig.22】


PERCENTAGE OF OLIGO dT
IL-2 EXPRESSION
INDUCED
NOT INDUCED
0
2
4
6
8
10
12
14
16
18
20
IL-2 228-242
IL-2 243-257
IL-2 198-212
IL-2 213-227
IL-2 77-91
IL-2 92-106
IL-2 287-301
IL-2 302-316
IL-2 342-356
IL-2 357-371
NONE

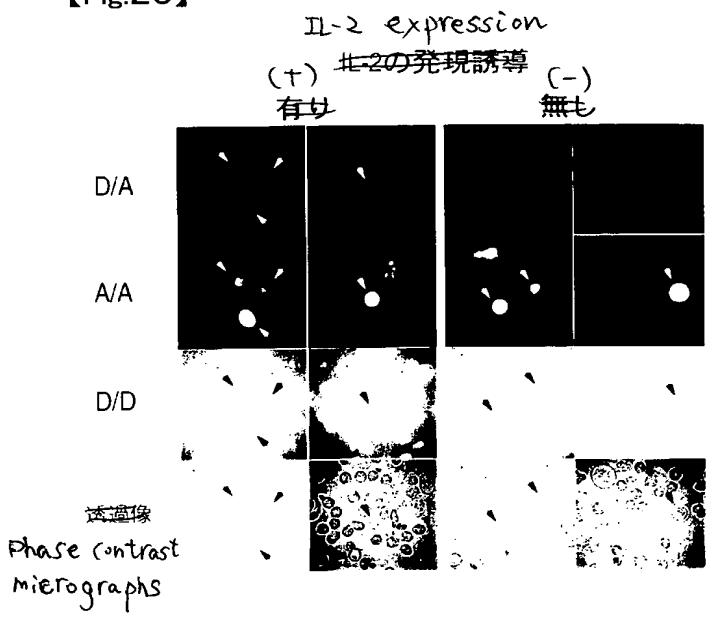
【Fig.23】


IL-2 EXPRESSION
PROBE
(+)
(-)
D/A
A/A
D/D
D/A
A/A
D/D
IL-2 228-242(D)
&
IL-2 243-257(A)
IL-2 198-212(D)
&
IL-2 213-227(A)
IL-2 77-91(D)
&
IL-2 92-106(A)
IL-2 287-301(D)
&
IL-2 302-316(A)
IL-2 342-356(D)
&
IL-2 357-371(A)
NONE
&
NONE

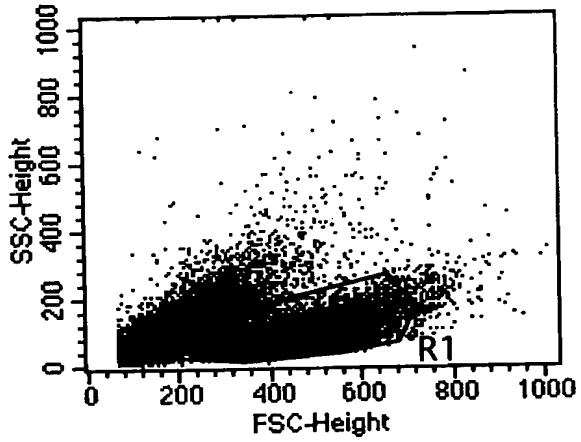
【Fig.24】


D/A / A/A (%)
0
5
10
15
20
25
30
35
40
45
50
IL-2 EXPRESSION INDUCTION
IL-2の発現誘導
INDUCED
有り
NOT INDUCED
無し
IL-2 228-242(D) & IL-2 243-257(A)
IL-2 198-212(D) & IL-2 213-227(A)
IL-2 77-91(D) & IL-2 92-106(A)
IL-2 287-301(D) & IL-2 302-316(A)
IL-2 342-356(D) & IL-2 357-371(A)
無し
NONE

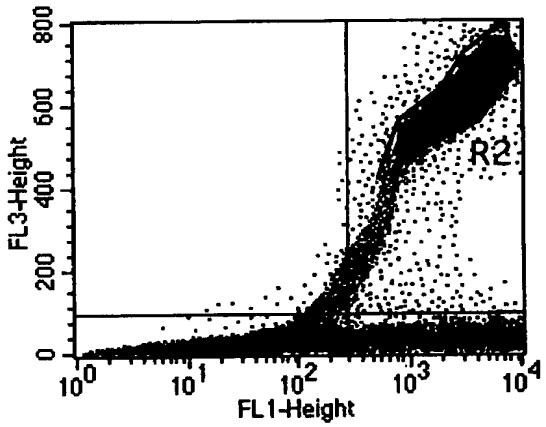
【Fig.25】


IL-2 expression
IL-2の発現誘導
(+)
有り
(-)
無し
D/A
A/A
D/D
位相像
Phase contrast micrographs

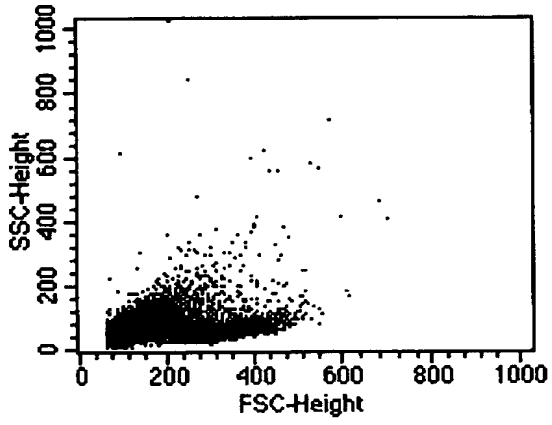
【Fig.26】



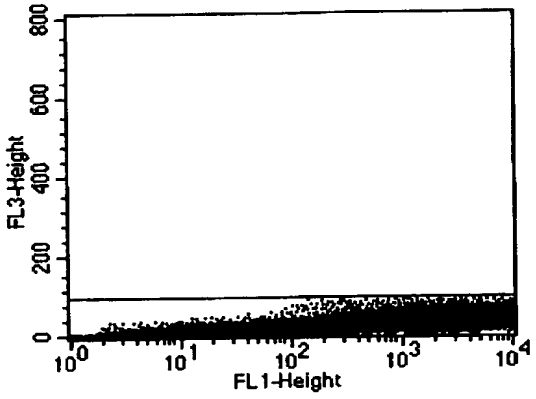
【Fig.27】



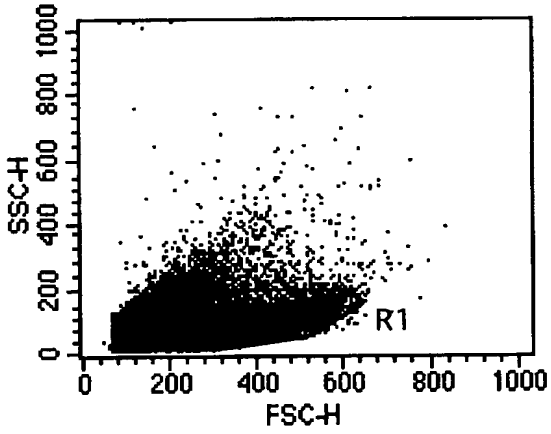
【Fig.28】



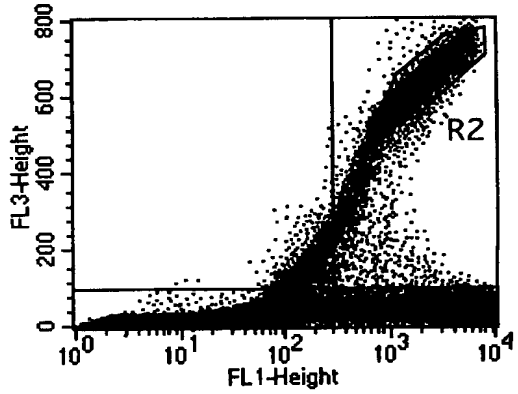
【Fig.29】



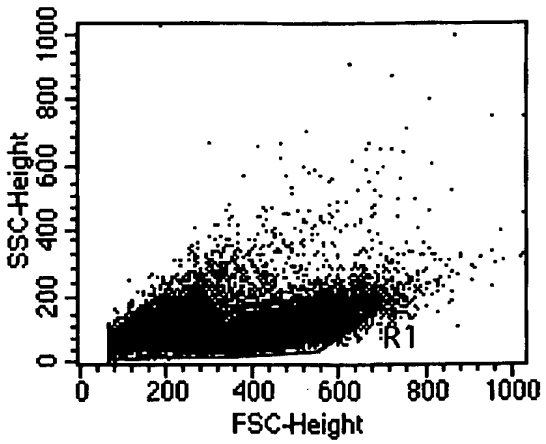
【Fig.30】



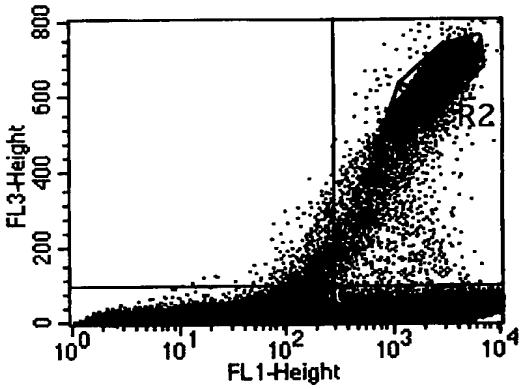
【Fig.31】



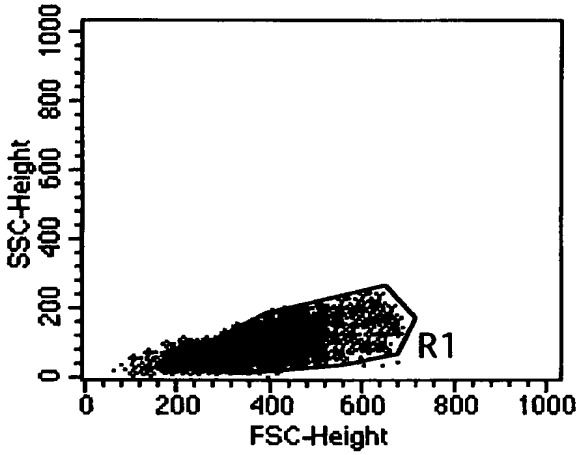
【Fig.32】



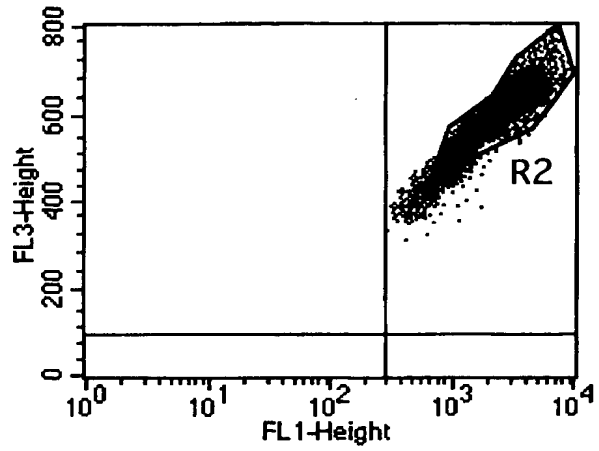
【Fig.33】



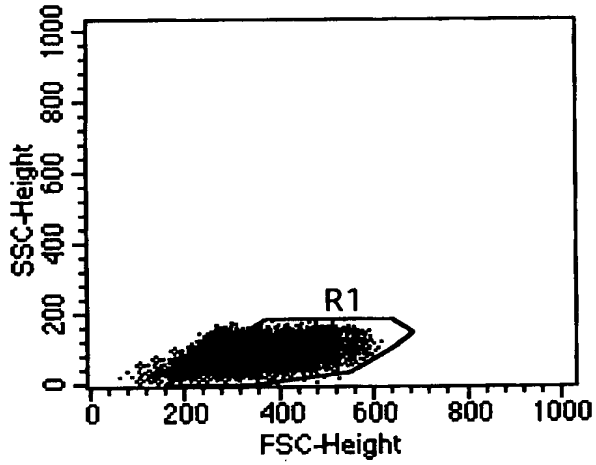
【Fig.34】



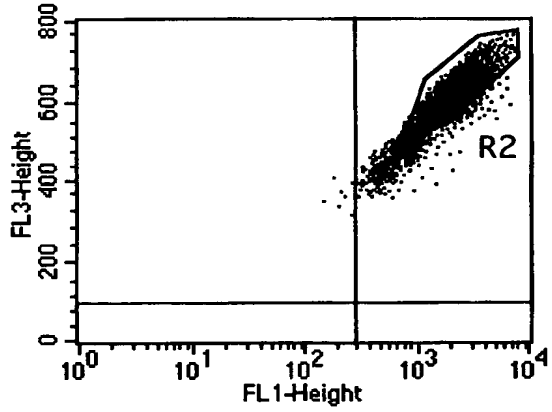
【Fig.35】



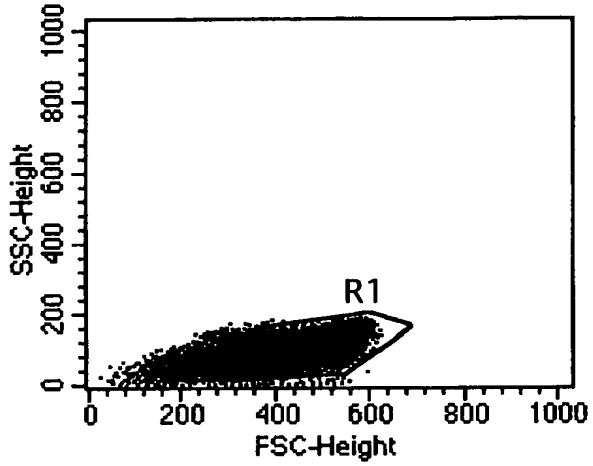
【Fig.36】



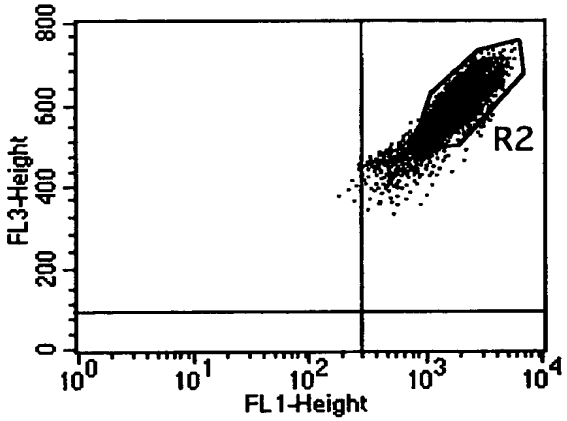
【Fig.37】



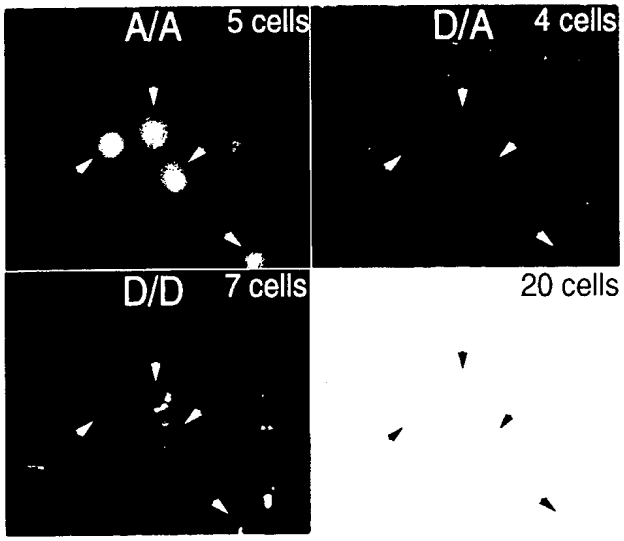
【Fig.38】



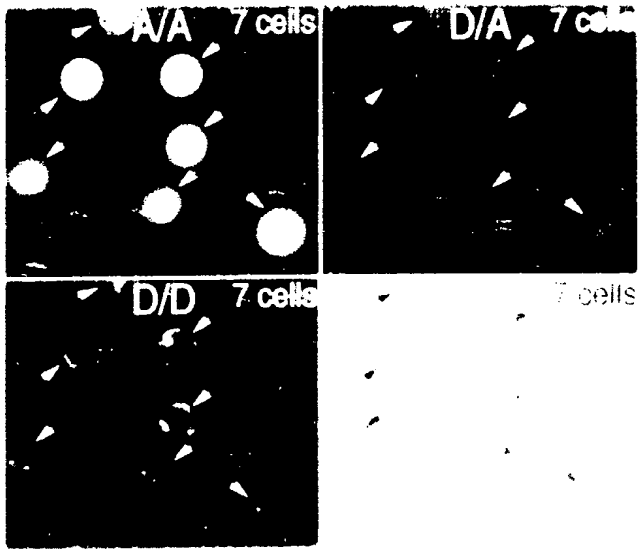
【Fig.39】



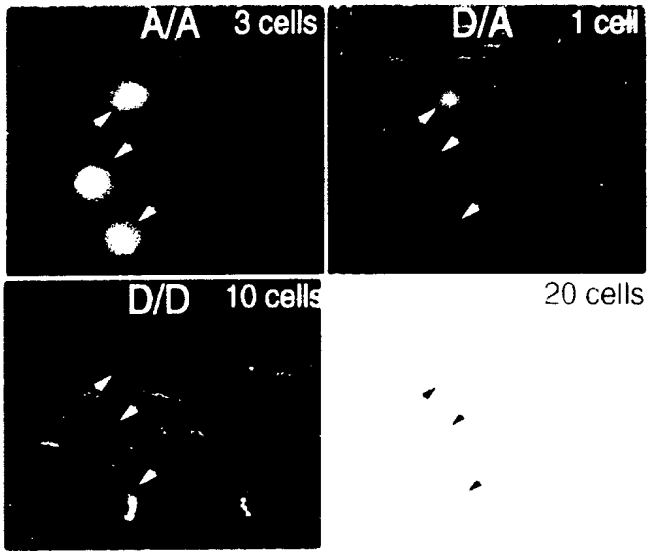
【Fig.40】



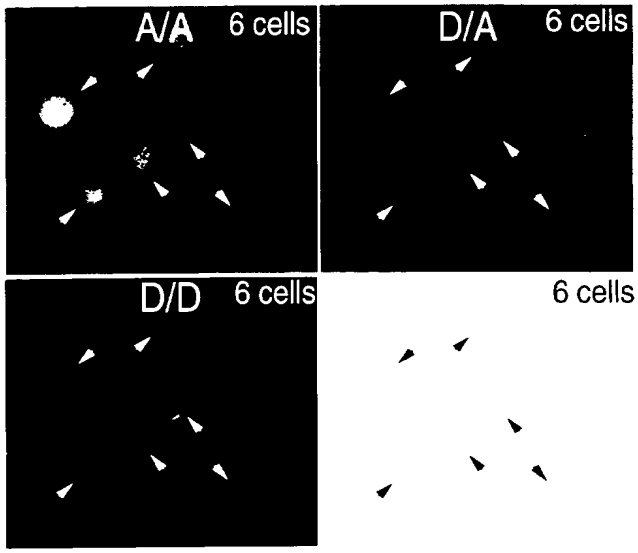
【Fig.41】



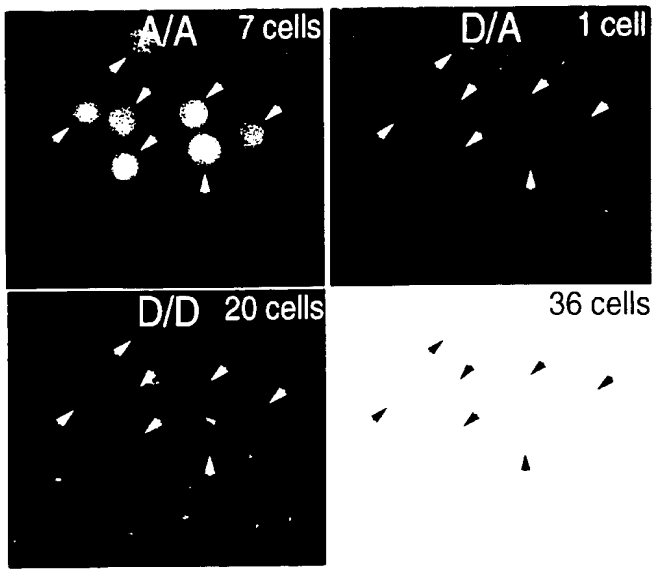
【Fig.42】



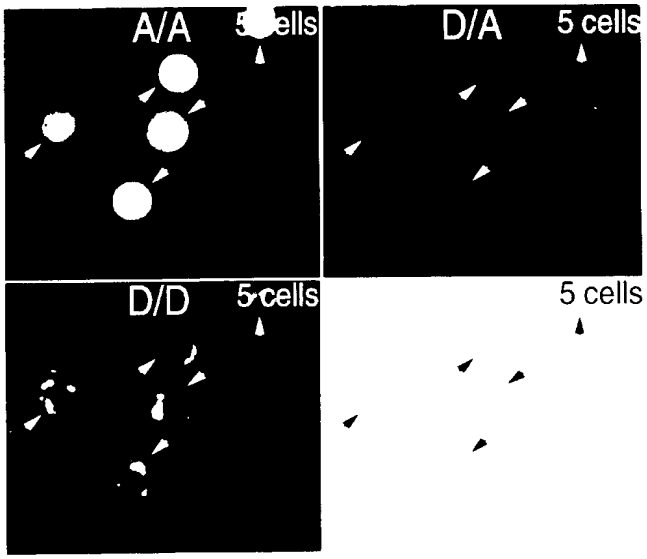
【Fig.43】



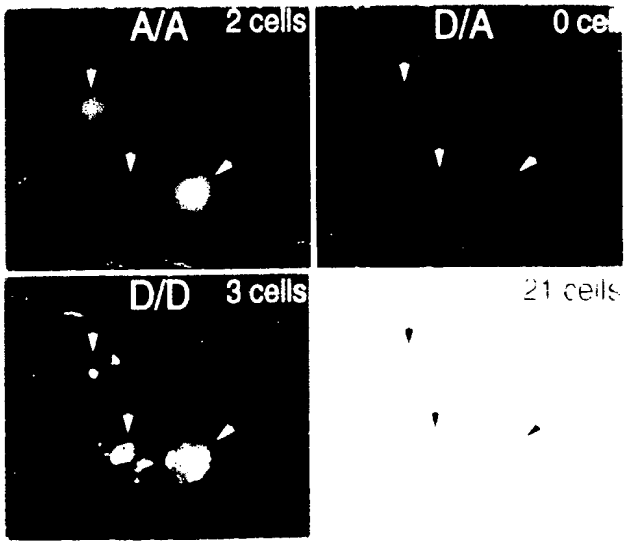
【Fig.44】



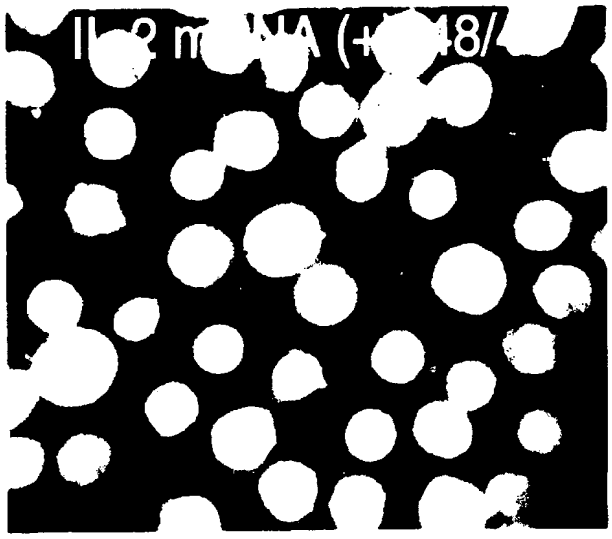
【Fig.45】



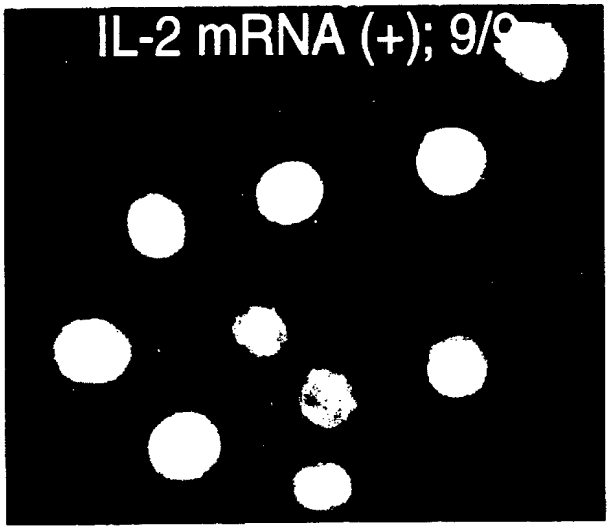
【Fig.46】



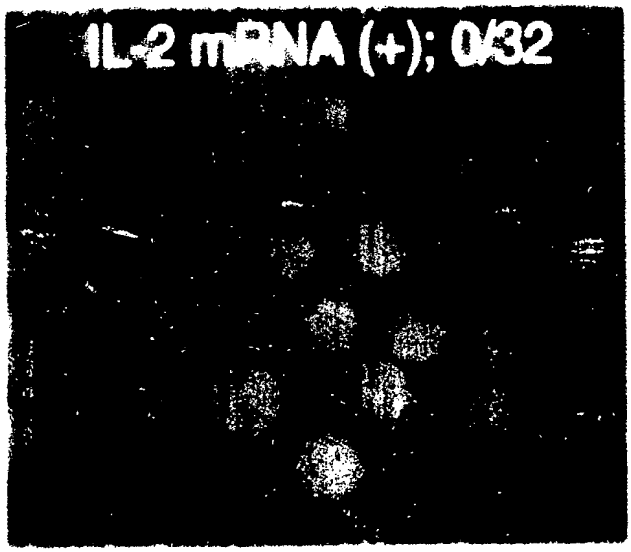
【Fig.47】



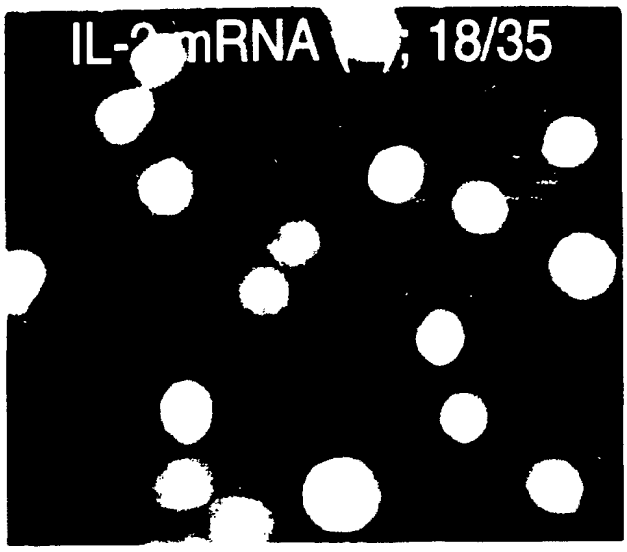
【Fig.48】



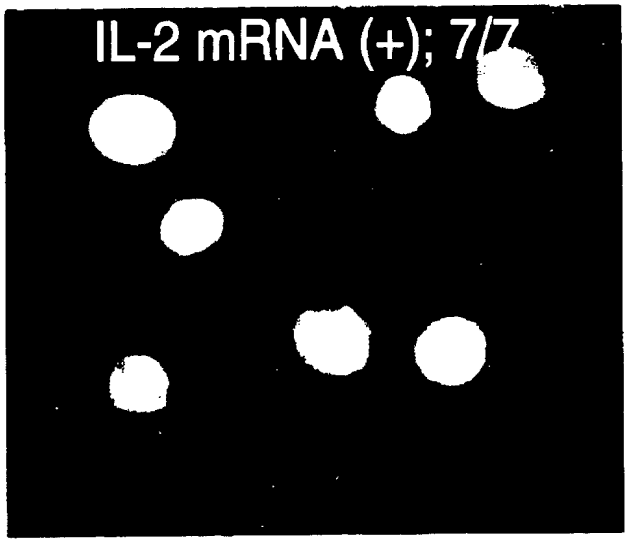
【Fig.49】



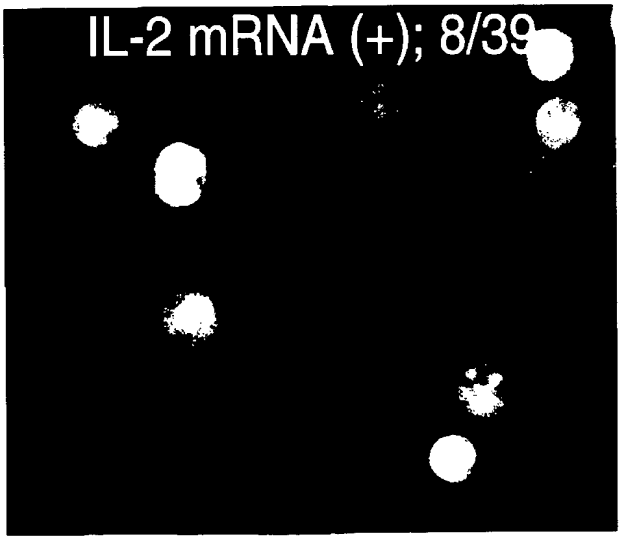
【Fig.50】



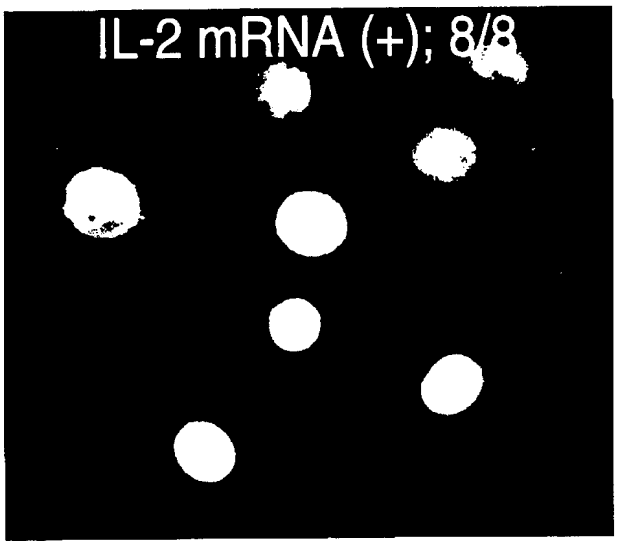
【Fig.51】



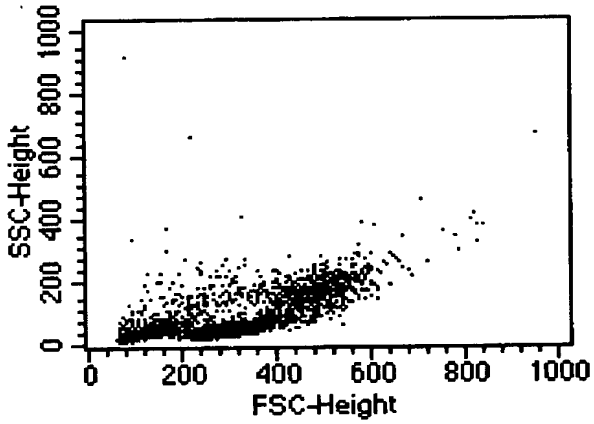
【Fig.52】



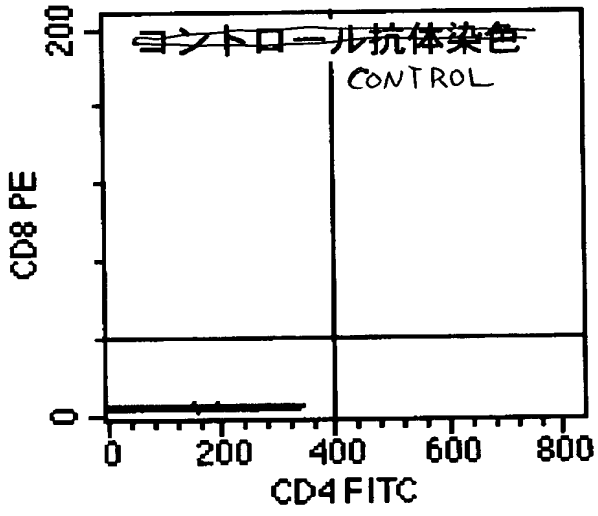
【Fig.53】



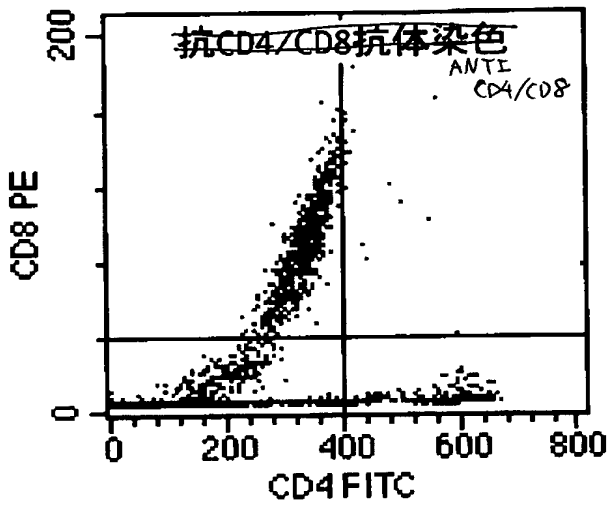
【Fig.54】



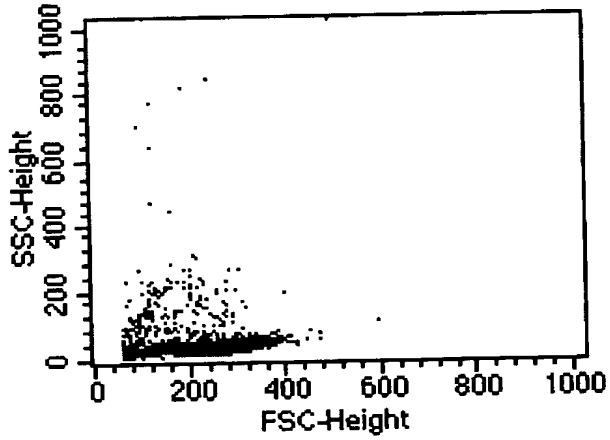
【Fig.55】



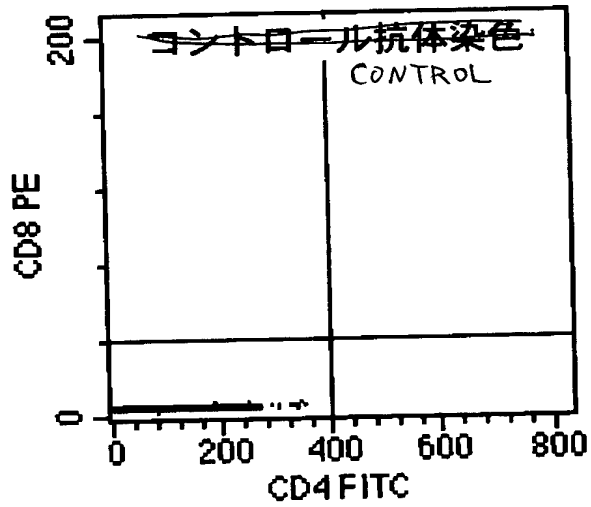
【Fig.56】



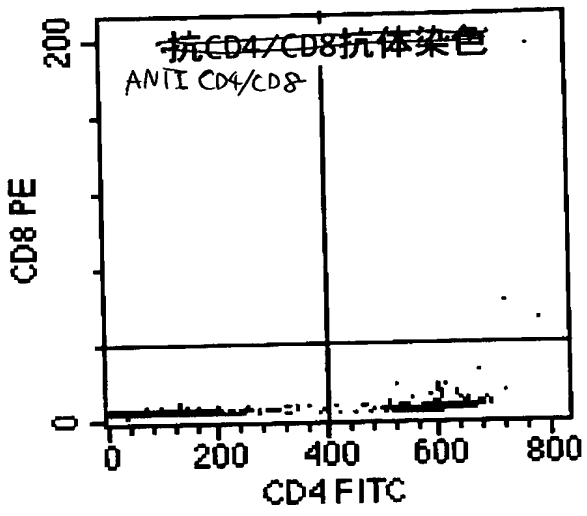
【Fig.57】



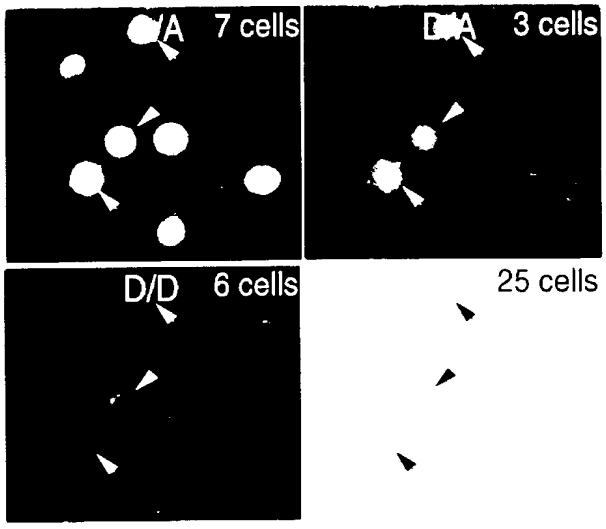
【Fig.58】



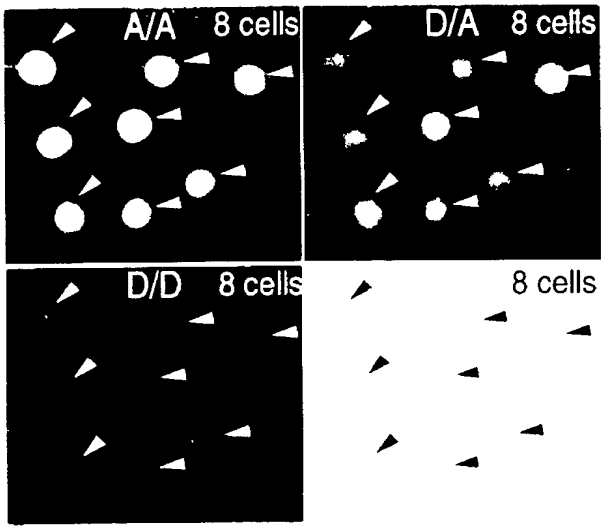
【Fig.59】



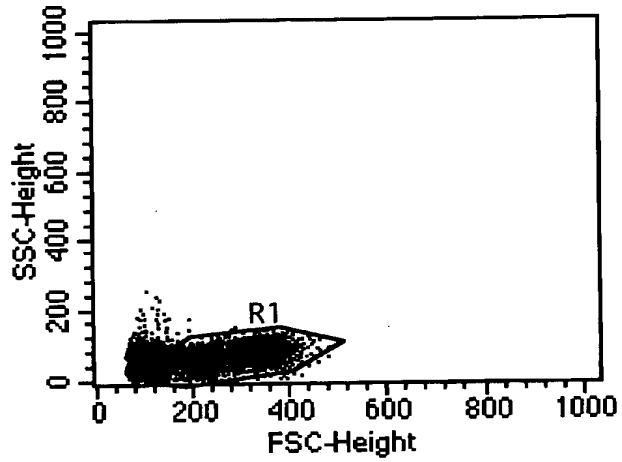
【Fig.60】



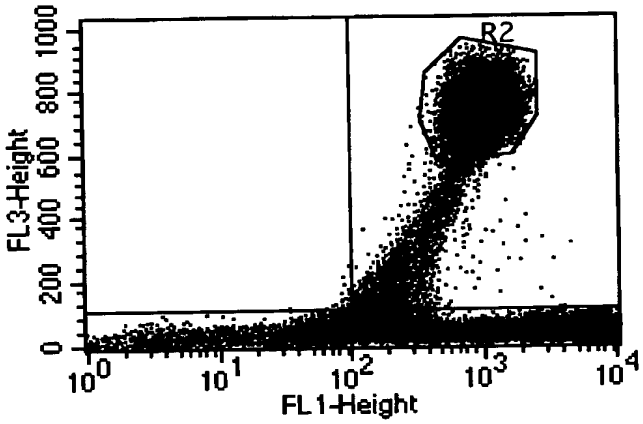
【Fig.61】



【Fig.62】



【Fig.63】



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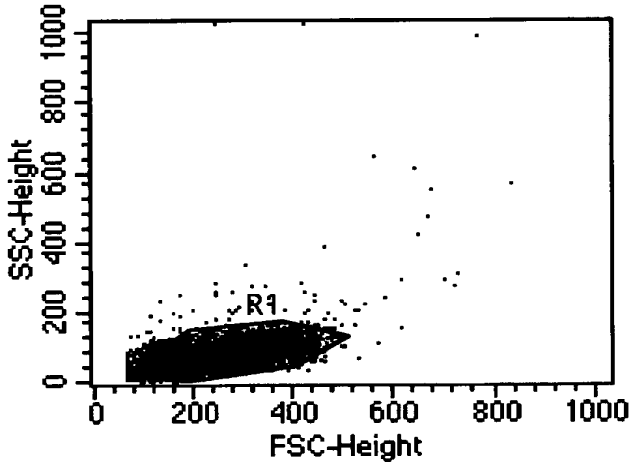
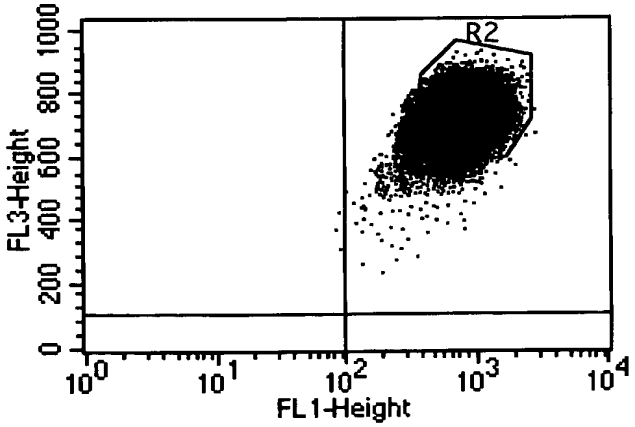
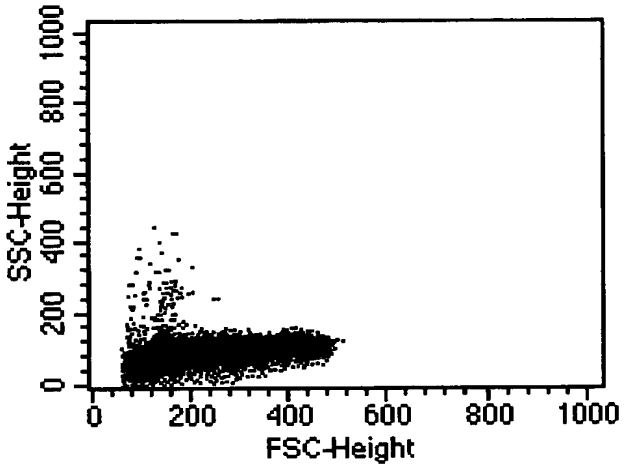


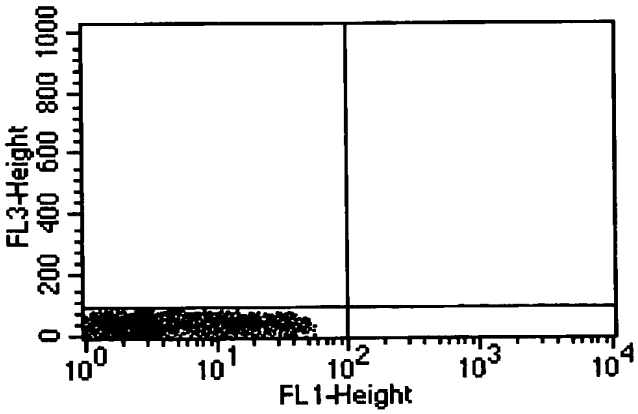
【Fig.65】



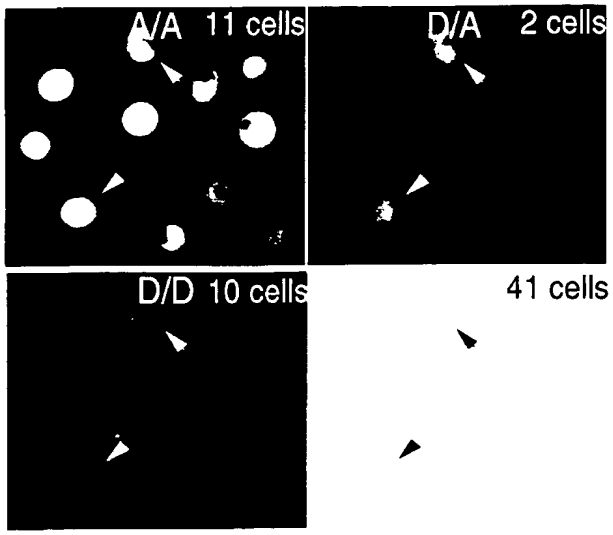
【Fig.66】



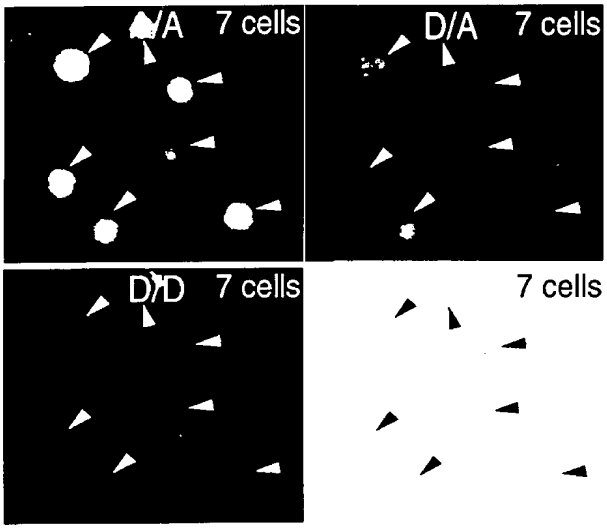
【Fig.67】



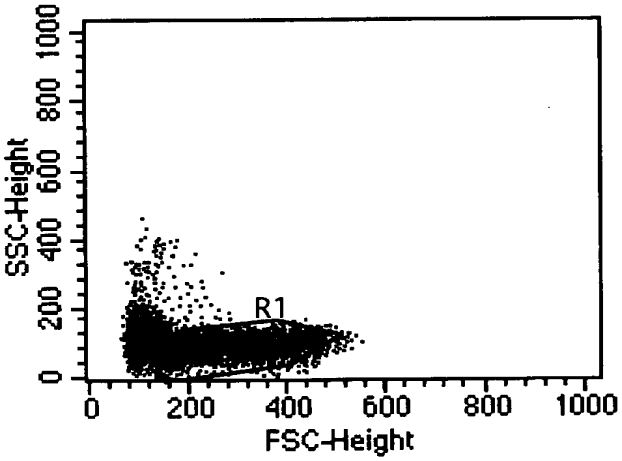
【Fig.68】



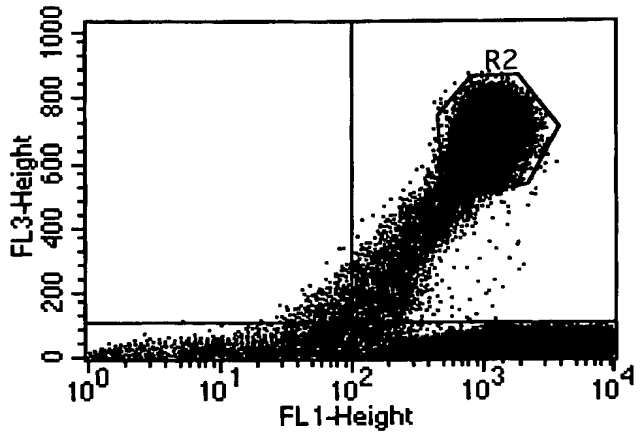
【Fig.69】



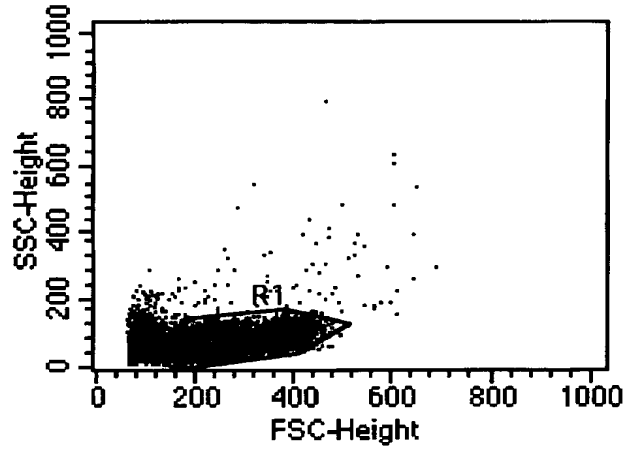
【Fig.70】



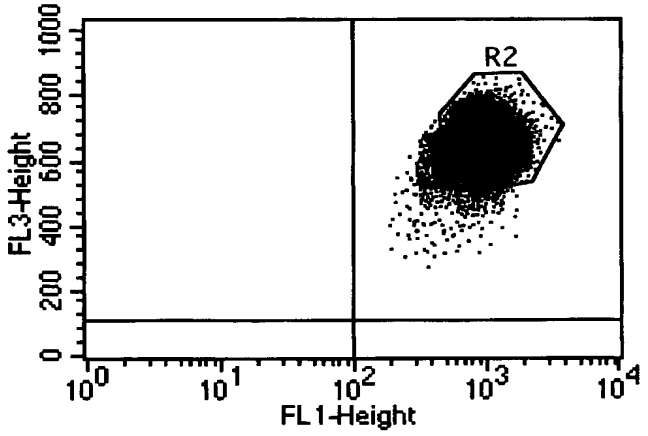
【Fig.71】



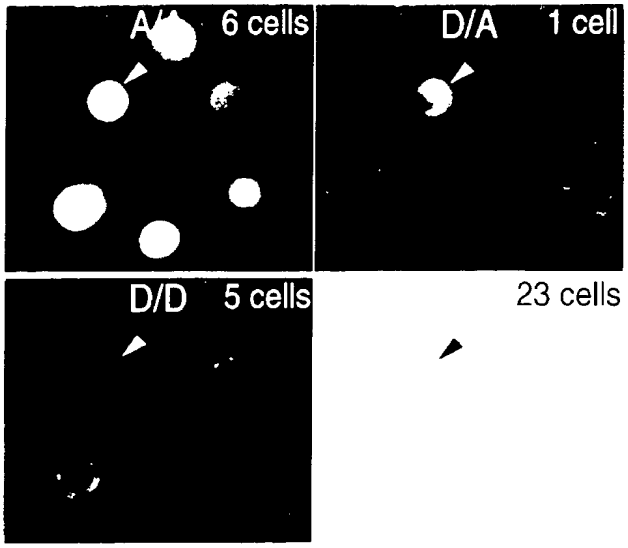
【Fig.72】



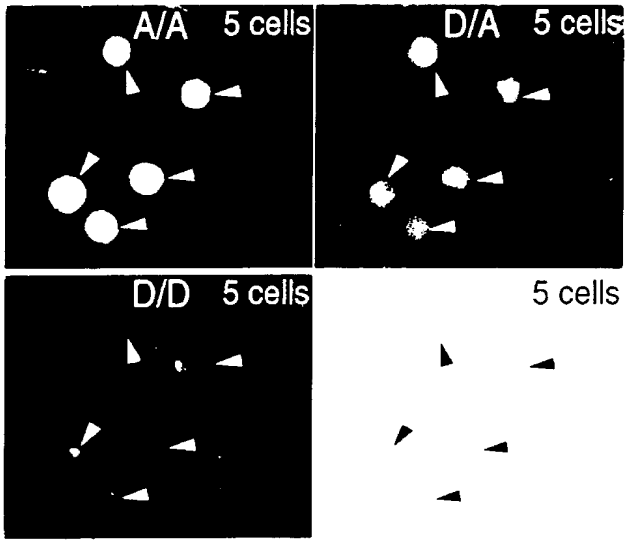
【Fig.73】



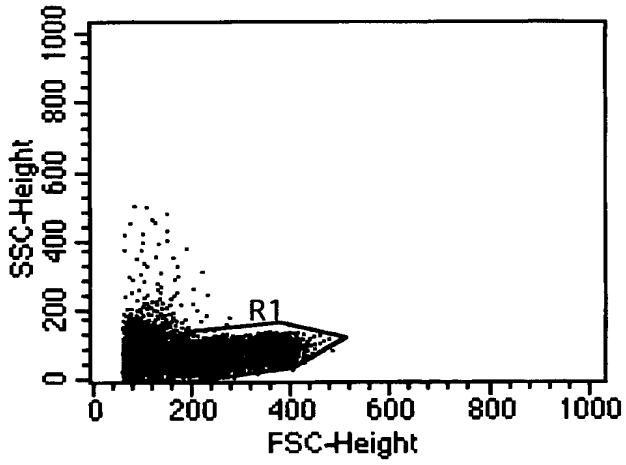
【Fig.74】



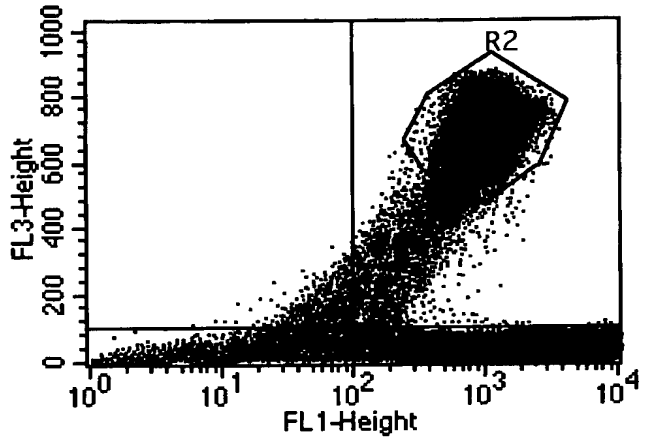
【Fig.75】



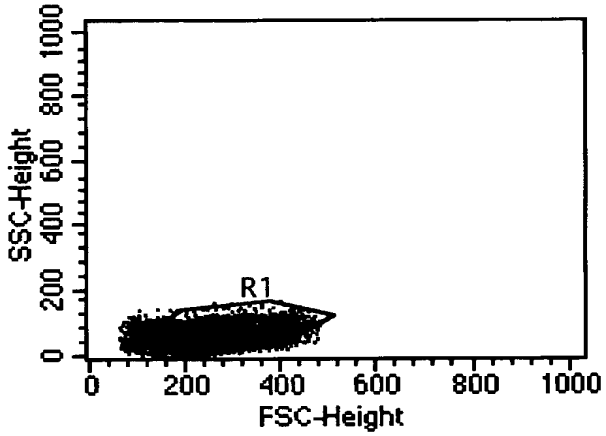
【Fig.76】



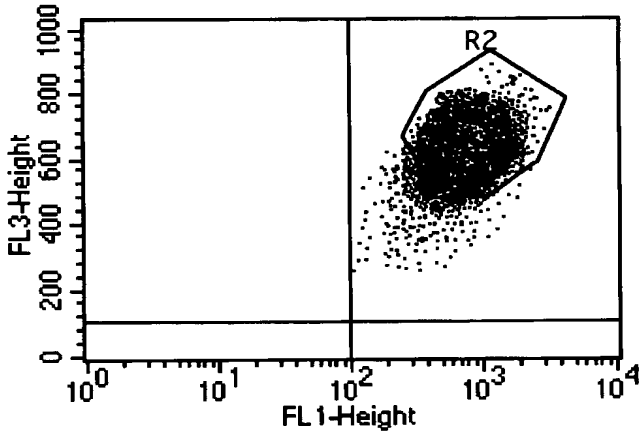
【Fig.77】



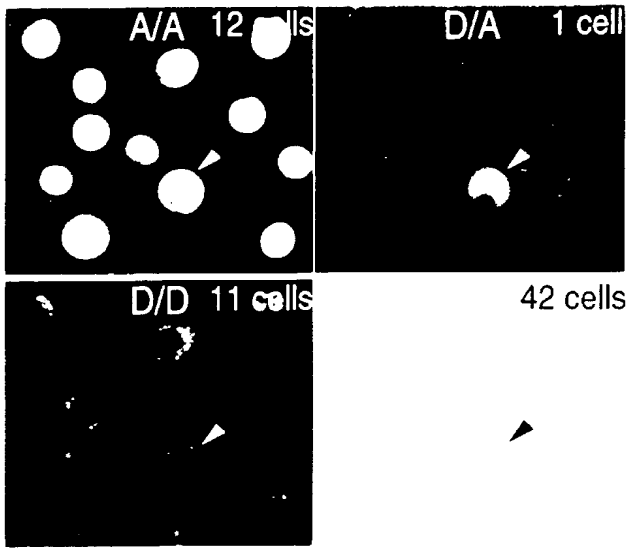
【Fig.78】



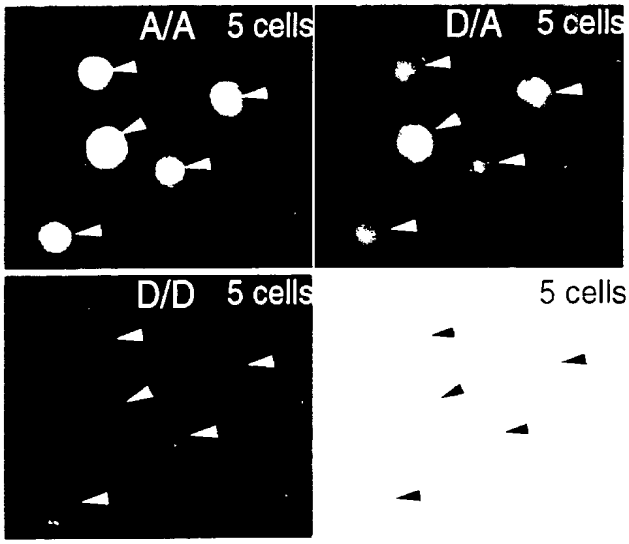
【Fig.79】



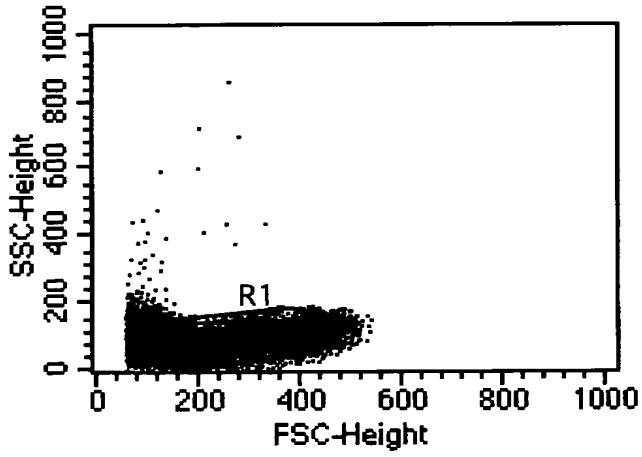
【Fig.80】



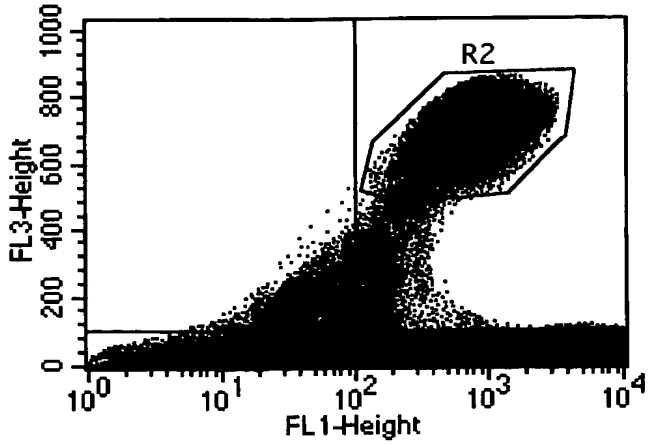
【Fig.81】



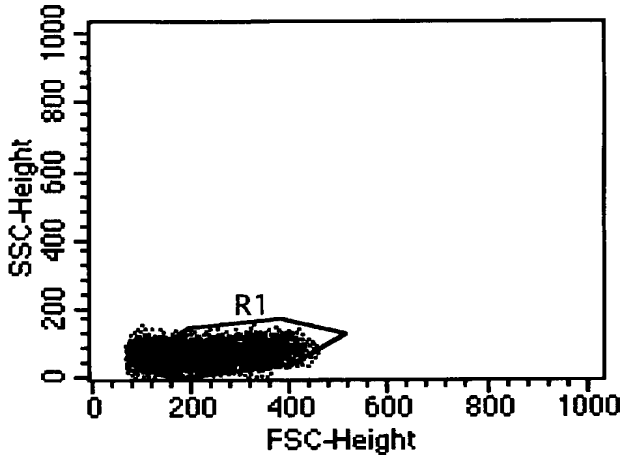
【Fig.82】



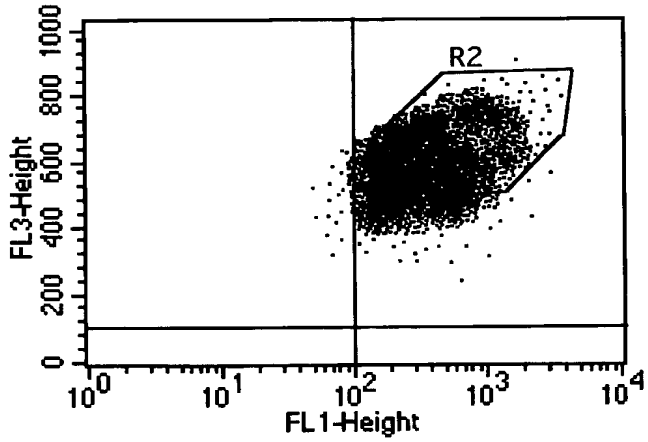
【Fig.83】



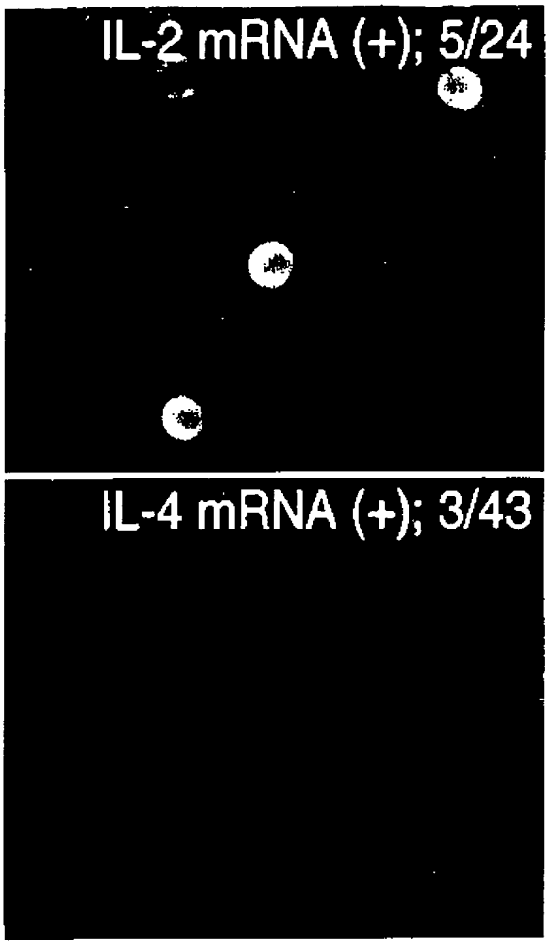
【Fig.84】



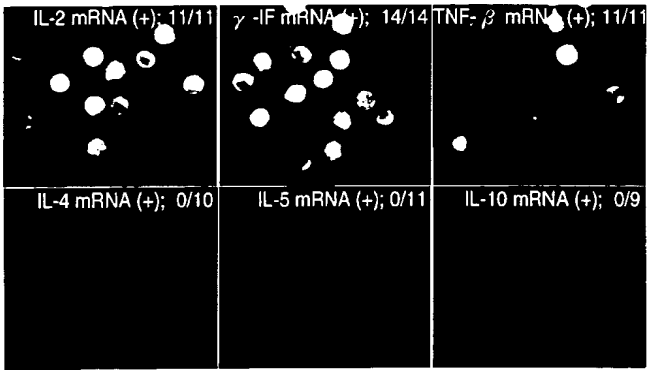
【Fig.85】



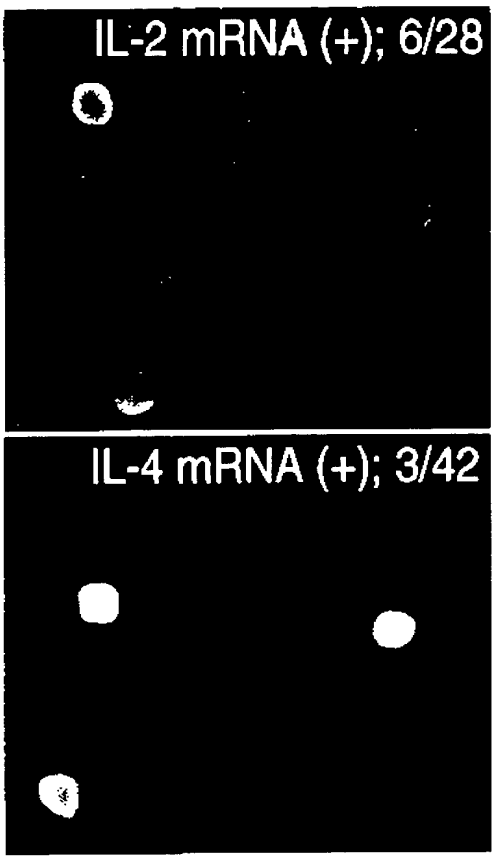
【Fig.86】


IL-2 mRNA (+); 5/24
IL-4 mRNA (+); 3/43

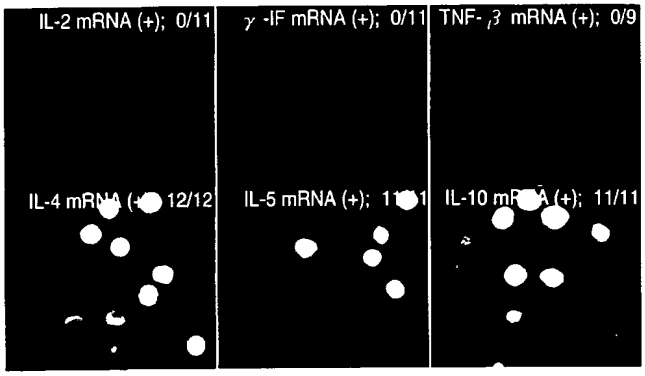
【Fig.87】


IL-2 mRNA (+); 11/11
γ -IF mRNA (+); 14/14
TNF-β mRNA (+); 11/11
IL-4 mRNA (+); 0/10
IL-5 mRNA (+); 0/11
IL-10 mRNA (+); 0/9

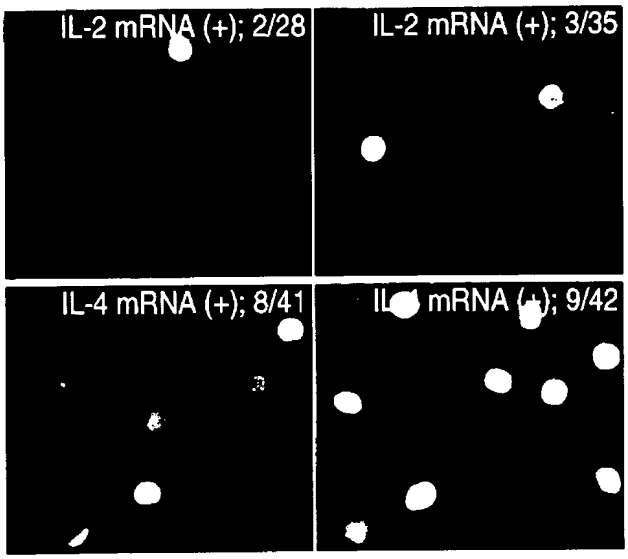
【Fig.88】


IL-2 mRNA (+); 6/28
IL-4 mRNA (+); 3/42

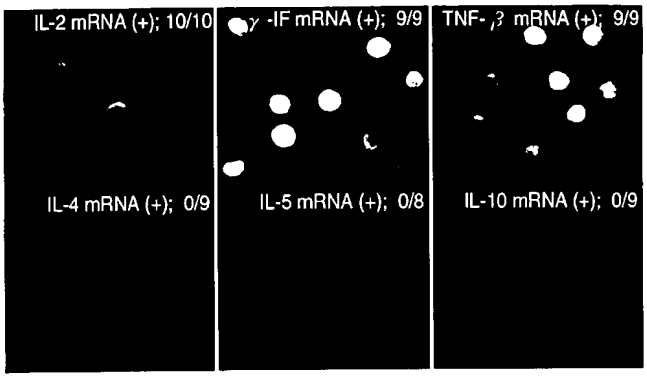
【Fig.89】


IL-2 mRNA (+); 0/11
γ -IF mRNA (+); 0/11
TNF-β mRNA (+); 0/9
IL-4 mRNA (+) 12/12
IL-5 mRNA (+); 11/11
IL-10 mRNA (+); 11/11

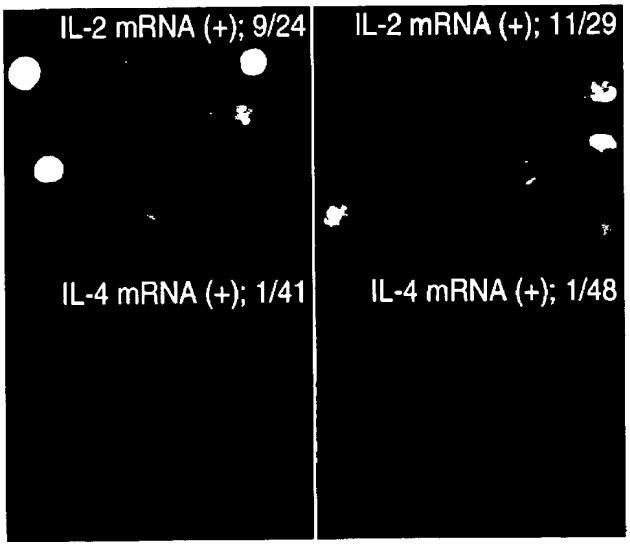
【Fig.90】



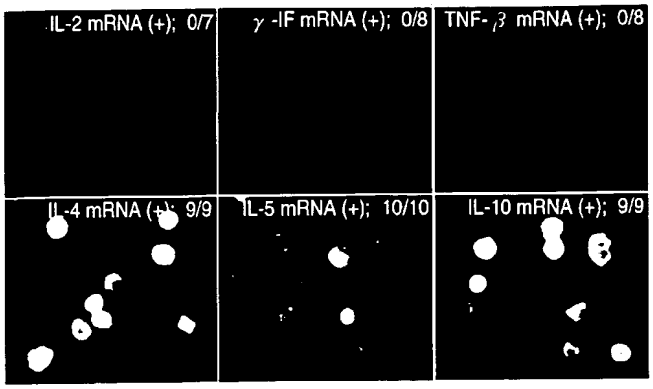
【Fig.91】



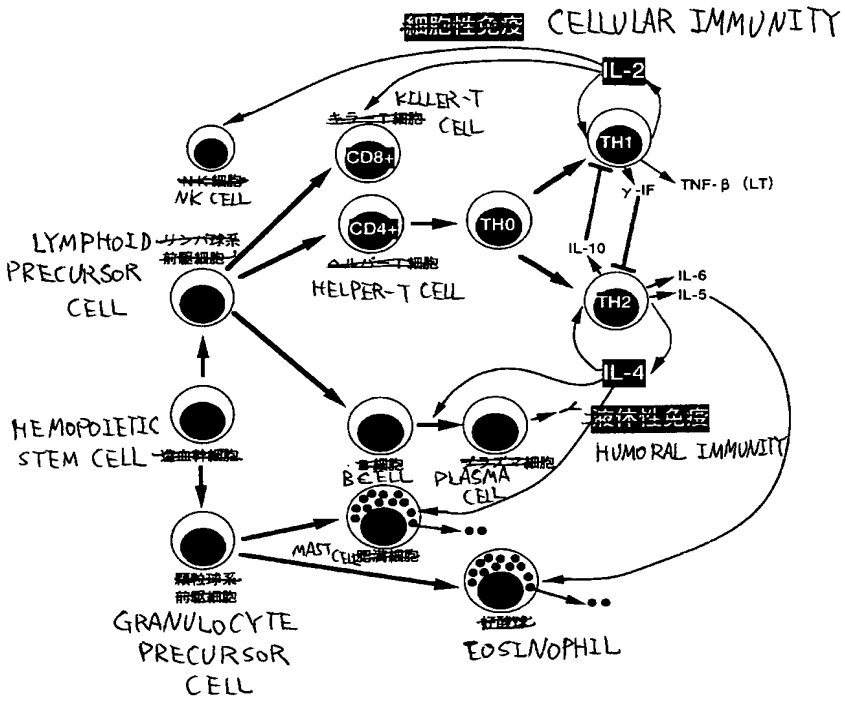
【Fig.92】



【Fig.93】


IL-2 mRNA (+); 0/7
γ -IF mRNA (+); 0/8
TNF-β mRNA (+); 0/8
IL-4 mRNA (+); 9/9
IL-5 mRNA (+); 10/10
IL-10 mRNA (+); 9/9

【Fig.94】


細胞性免疫 CELLULAR IMMUNITY
IL-2
KILLER-T CELL
CD8+
TH1
NK CELL
γ-IF
TNF-β (LT)
LYMPHOID PRECURSOR CELL
CD4+
TH0
IL-10
HELPER-T CELL
IL-6
IL-5
TH2
IL-4
液体性免疫
HUMORAL IMMUNITY
HEMOPOIETIC STEM CELL
B CELL
PLASMA CELL
MAST CELL
GRANULOCYTE PRECURSOR CELL
EOSINOPHIL

[NAME OF DOCUMENT] Abstract

[ABSTRACT]

[OBJECT] To provide a separation method which allows one to selectively separate and obtain the objective cells, that is, the cells which have expressed a specific gene, when there are no cell surface molecules usable as markers in the cell, or when the cell surface molecules cannot be distinguished from each other even if they are present in the cell, or even when the molecules to be the markers are liberated into the extracellular fluid.

[SOLUTION] A method for selectively separating live cells which have expressed mRNA comprising a first step of introducing a marker capable of labeling mRNA into cells in a live cell group containing live cells which have expressed a specific mRNA, a second step of labeling said mRNA with said marker to obtain a live cell group containing live cells having the labeled mRNA, and a third step of detecting said labeled mRNA to identify the live cells having said labeled mRNA and separating the identified live cells selectively from said live cell group obtained in said second step.

[SELECTED DRAWING] none